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"Study of transcriptional alterations in Amyotrophic Lateral Sclerosis"

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a progressive fatal neuromuscular disease characterized by selective motoneurons loss. Since mutations in TARDBP and FUS genes were discovered to cause familial form of ALS and TDP-43 and FUS proteins play important roles in RNA metabolism, transcriptional alterations emerged as potential pathogenic mechanism. RNA metabolism include several aspects of RNA regulation such as RNA transcription, maturations and regulation.

In this study we have investigated two different aspects of RNA metabolism: the first one concerns to microRNAs (miRNA) which regulate translation of several mRNAs, and the second one is related to a specific muscular and neuronal transcription factor potential involved in ALS. First, we have assessed any selected miRNAs with neuronal functions in human neuroblastoma cell lines expressing the pathological SOD1(G93A) mutation and we found a small group of altered miRNAs. Subsequently, we explored these miRNAs in the spinal cord of transgenic SOD1(G93A) mice identified a panel of targets commonly altered in SOD1 ALS models. Furthermore, we assessed the expression levels of a panel of selected miRNAs in circulating cells obtain from patients affected by sporadic ALS form (sALS). This approach let us to identify two microRNAs (miR129-5p and miR200c) that were up-regulated in both SOD1 ALS models and in blood cells of patients with sporadic form of disease, evidencing two possible events potentially involved in the pathogenesis of both the sporadic and the familial form of ALS. Moreover, we also identified HuD protein as a potential molecular target of miR129-5p; this protein has been previously reported to play a role in neuronal plasticity and in recovery from axonal injury. Indeed, in a cell line stably overexpressing mir129-5p we found a reduction in neurite outgrowth and decreased expression levels of differentiation markers with

respect to control cells. Taken together these data strongly suggest that microRNAs play a role in ALS pathogenesis and in particular that mir129-5p can affect neuronal plasticity by modulating HuD levels.

In the second part of the study we investigated the possible involvement of two members of myocyte enhancer factor 2 (MEF2) family in the pathogenesis of ALS. MEF2D and MEF2C are transcriptional factors playing crucial roles both in muscle and in neuron development and maintenance. We have performed gene expression analysis in peripheral blood mononuclear cells (PBMCs), we showed a strong increased in MEF2D and MEF2C levels both in sporadic and in familial ALS (SOD1+) patients and a direct correlation between MEF2D and MEF2C mRNA levels was observed in patients and controls. Although protein levels were unchanged, a different pattern of distribution for MEF2D-MEF2C proteins in patient cells was found, suggesting a possible lack of their function. To evaluate the transcriptional activity of MEF2 proteins mRNA levels of their downstream targets BDNF, KLF6, RUFY3 and NPEPPS were assessed. Our results showed a significant down-regulation of BDNF, KLF6 and RUFY3 levels confirming that transcriptional activity of both MEF2D and MEF2C isoforms was altered in sporadic and familial ALS patients. In conclusion, our results evidenced a systemic alteration of MEF2D and MEF2C pathways in ALS patients independently from the presence of SOD1 gene mutations, highlighting a possible common feature between the sporadic and the familiar form of disease which are characterized by a different clinical phenotype and pathological hallmarks. In conclusion, the interconnections between these two topics will be discussed to delineate a possible common regulatory mechanism potentially involved in ALS.

Key Words: amyotrophic lateral sclerosis; microRNA; miR129-5p; miR200c; HuD; SOD1 fALS; MEF2D; MEF2C; BDNF; KLF6; RUFY3.

Chapter 1 - General Introduction

1.1 Amyotrophic Lateral Sclerosis (ALS)

1.1.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neuro-degenerative disease caused by the loss of both upper and lower motor neurons. ALS is the most common adult onset motor neuron disease with an incidence of 2 individuals per 100,000 and a prevalence of 6-8 per 100,000 of the total population. Most people developing ALS are between the ages of 40 and 70, with an average age of 55 at the time of diagnosis. ALS is 20% more common in men than in women. However, with increasing age, the incidence of ALS becomes similar in the two sexes. Affected individuals develop progressive muscle weakness with death due to respiratory failure typically within 3-5 years after symptom onset, with great individual variation in the pathology progression rate (Strong et al., 2005). It spares cognitive ability, sensation, and autonomic nervous functions, and only in a few cases do patients also develop frontotemporal dementia (FTD). Most of the ALS cases occur in sporadic (sALS) forms, while about 10–15% of the cases are familial (fALS) forms, clinically indistinguishable from sALS (Bendotti et al., 2012).

1.1.2 Genetics of ALS

The high phenotypic heterogeneity of the clinical face of ALS pales in comparison to its biological heterogeneity, reflected in part by the extensive number of genetic variants of the disease that are now recognized (Table 1). For almost 15 years, the only gene clearly associated with fALS was the *Cu-Zn superoxide dismutase 1* (SOD1) gene (Rosen et al.,

1993), which accounts for 20% of fALS cases. The identification of SOD1 mutations in 1993 ushered in the molecular era of ALS research, and significant insight into ALS pathogenesis has been gained through identification of pathways directly affected by the toxicity of mutant SOD1. Two main discoveries in mutant SOD1-mediated ALS in rodent models are the demonstration that the SOD1 protein aggregates produce a toxic gain of function that causes neuronal loss, and the disease can spread in a non-cell-autonomous manner in the nervous system (Boillée et al., 2006; Yamanaka et al., 2008). A major shift in our understanding of ALS pathogenesis occurred in 2006 with the identification of a 43-kDa transactive response (TAR) DNA binding protein (TDP-43) as a key pathological substrate of cellular inclusions in sALS and non-SOD1 fALS, and frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) (Neumann et al., 2006).

Table 1
Genes associated with high risk of ALS-causative genes.

Locus	Chromosome	Gene ID	Protein/function	Evidence	Inheritance	Mutation	Diagnosis	Onset	Positive replication studies	References
ALS1	21q22.11	SOD1	Cu/Zn superoxide dismutase 1, soluble/Oxidative stress	Linkage, Sanger	AD, AR	SNV	ALS, PMA	Adult	Yes	(Rosen et al., 1993)
ALS2	2q33.2	ALSO	Alsin/Rho guanine nucleotide exchange factors	Linkage, Sanger	AR	SNV	ALS, pLS, HSP	Juvenile	Yes	(Hadano et al., 2001; Yang et al., 2001)
ALS4	9q34.13	SETX	Senataxin/RNA/DNA helicase	Linkage, Sanger	AD	SNV	ALS, AOA2	Juvenile	Yes	(Chen et al., 2004)
ALS5	15q21.1	SPG11	Spatacin/transmembrane protein	Linkage, Sanger	AR	SNV	ALS, HSP	Juvenile	Yes	(Hentati et al., 1998; Orlacchio et al., 2010)
ALS6	16p11.2	FUS	Fused in sarcoma/RNA-binding protein, DNA repair; exon splicing	Linkage, Sanger	AD	SNV	ALS, ALS-FTD	Adult	Yes	(Kwiatkowski et al., 2009; Vance et al., 2000)
ALS7	20p13	Unknown	Unknown	Linkage	AD	—	ALS	Adult	No	(Sapp et al., 2003)
ALS8	20q13.33	VAPB	Vesicle-associated membrane protein-associated protein B/vesicular trafficking	Linkage, Sanger	AD	SNV	ALS, SMA	Adult	Yes	(Nishimura et al., 2004a, 2004b)
ALS10	1p36.22	TARDBP	TAR DNA binding protein 43/transcriptional repressor, splicing regulation	Linkage, Sanger	AD	SNV	ALS, ALS-FTD	Adult	Yes	(Kabashi et al., 2008; Sreedharan et al., 2008)
ALS12	10p13	OPTN	Optineurin/ocular tension, membrane and vesicle trafficking	Homozygosity mapping, Sanger	AD/AR	SNV	ALS, POAG	Adult	Yes	(Miyayama et al., 2010)
ALS14	9p13.3	VCP	Valosin-containing protein/ATP-binding protein, vesicle transport and fusion	WES linkage, Sanger	AD	SNV	ALS, ALS-FTD, FTD, IBMPPD	Adult	No	(Johnson et al., 2010)
ALS15	Xp11.21	UBQLN2	Ubiquilin 2/ubiquitination, degradation	Linkage, Sanger	X-linked AR	SNV	ALS, ALS-FTD	Juvenile	Yes	(Deng et al., 2011)
ALS16	9p13.3	SIGMAR1	Sigma non-opioid intracellular receptor 1/endoplasmic reticulum chaperone	Homozygosity mapping, Sanger	AR	SNV	ALS, FTD	Juvenile	No	(Al-Saif et al., 2011)
	12q24.11	DAO	D-amino-acid oxidase/unknown	Linkage, Sanger	AD	SNV	ALS	Adult	No	(Mitchell et al., 2010)
	17p13.2	PEN1	Profilin 1/lacrin binding protein, actin polymerization	Linkage, WES, Sanger	AD	SNV	ALS	Adult	No	(Wu et al., 2012)
	7p15.2/12q13.3	hnRNPA2B1/A1	Heterogenous nuclear ribonucleoprotein/n mRNA processing, metabolism & transport	Linkage, WES, Sanger	AD	SNV	ALS, IBMPPD	Adult	No	(Kim et al., 2013)
ALS-FTD1	9q21-q22	Unknown	Unknown	Linkage	AD	SNV	ALS, ALS-FTD, FTD	Adult	No	(Hosler et al., 2000)
ALS-FTD2	9p21.2	C9orf72	Chromosome 9 open reading frame 72/unknown	Linkage, GWAS, RP-PCR Southern blot	AD, sporadic	G ₄ C ₂ repeat	ALS, ALS-FTD, FTD	Adult	Yes	(DeJesus-Hernandez et al., 2011; Gijssenick et al., 2012; Lahsivirta et al., 2010; Morita et al., 2006; Renton et al., 2011; Shatunov et al., 2010; Valdmanis et al., 2007; van Es et al., 2009; Vance et al., 2006)

ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; WES, whole exome sequencing; GWAS, genome wide association study; RP-PCR, repeat-primed polymerase chain reaction; AD, autosomal dominant; AR, autosomal recessive; SNV, single nucleotide variation; PMA, progressive muscular atrophy; HSP, hereditary spastic paraparesia; PLs, primary lateral sclerosis; AOA2, ataxia-ocular apraxia 2; SMA, spinal muscular atrophy; IBMPPD, inclusion body myopathy with Paget's disease and frontotemporal dementia.

Table 1. ALS-causing genes as appeared in (Leblond et al., 2014)

It was soon followed in 2008 by the successful discovery of dominant mutations in the TDP-43 gene as a primary cause of primary cause of ALS (Sreedharan et al., 2008), thus providing the proof of principle that aberrant TDP-43 can trigger neuronal degeneration and cause ALS. A total of 44 TDP-43 mutations (Da Cruz and Cleveland, 2011) have since been reported in ALS patients with or without an apparent family history, accounting for approximately 5% of fALS4 , and rare sALS cases (Da Cruz and Cleveland, 2011; Kabashi et al., 2008; Mackenzie et al., 2010; Sreedharan et al., 2008). The identification of TDP-43 mutations was followed in tandem by the discovery of mutations in another RNA/ DNA-binding protein, FUS/TLS (fused in sarcoma and translocated in liposarcoma) in 2009, as a primary cause of fALS (Kwiatkowski et al., 2009). In a period of less than 3 years, at least 50 different FUS mutations have been described in fALS, accounting for another 4% of fALS (Da Cruz and Cleveland, 2011; Lanson and Pandey, 2012) and rare sALS (Lanson and Pandey, 2012; Mackenzie et al., 2010) cases. Both in TDP-43- and FUS -linked ALS, the age and site of disease-onset and clinical phenotype are variable, as in sALS, and incomplete penetrance has been documented for several of these mutations (Da Cruz and Cleveland, 2011; Kabashi et al., 2008; Mackenzie et al., 2010; Sreedharan et al., 2008). Most patients with TDP-43 and FUS gene mutations develop the classical ALS phenotype, with characteristic pathological features of the disease. A careful review of other rare forms of fALS reveals that mutations in several other genes that encode RNA-processing proteins, including survival motor neuron (Lefebvre et al., 1995), senataxin (Chen et al., 2004), angiogenin (Greenway et al., 2006), optineurin (Maruyama et al., 2010), and TAF15 (Couthouis et al., 2011; Malpass, 2012) can also give rise to motor neuron degeneration (Table 1, see review (Ticozzi et al., 2011). A hexanucleotide repeat expansion in the noncoding region

(intron) of *C9orf72* gene has recently emerged as a relatively common genetic form among fALS cases (Renton et al., 2011). It has been shown to lead to altered regulation of RNA splicing, mRNA translation, and mRNA stability, which contribute to the disease processes. Additionally, genes that encode proteins involved in proteosomic or autophagic aggregate clearance and disposal, including UBQLN2 (Deng et al., 2011) and SQSTM1 (Fecto et al., 2011), are linked to fALS. Taken together, the emerging concept that RNA-processing abnormality and protein aggregate accumulation are crucial elements in ALS is taking a tangible shape.

1.1.3 Cu-Zn superoxide dismutase 1 (SOD1)

SOD1 mutations were the most frequently identified cause of FALS, before the discovered of *C9orf72* expansion, accounting for ~20% of all patients. In 1993, Rosen et al. described eleven disease-associated mutations in the SOD1 gene, encoding for the Cu/Zn superoxide dismutase, a cytoplasmic enzyme responsible for the catabolism of superoxide radicals to hydrogen peroxide and molecular oxygen (McCord and Fridovich, 1969). SOD1 is ubiquitously expressed, highly conserved, and represent ~1% of all cytoplasmic proteins. To date, 119 types of *SOD1* mutations have been discovered but the induction of the disease is thought to depend upon few common pathways. The two principal critical features of SOD1 mediated toxicity are toxic gain of function and that the pathogenesis of the ubiquitously expressed mutant SOD1 is a non-cell autonomous process. This means that disease onset is driven by mutant protein synthesized inside motor neurons. Mutated SOD1 protein interacts specifically with neurofilament-light chain mRNA and the dynein/dynactin complex, thus inducing cytoskeletal defects or altering axonal transport. Furthermore has increased tendency to form aggregate-prone

monomers, and the degree of instability correlates inversely with length of survival; this suggest that increased propensity to aggregate may be the unifying common denominator for the 119 diverse *SOD1* mutations (Andersen, 2006). Some investigators (Bosco et al., 2010b) found misfolded SOD1 in motor neurons in a subset of patients with sALS without SOD1 mutations, thus suggesting a role for wild-type SOD1 in sALS, possibly after secondary (oxidative) modification (Robberecht and Philips, 2013). Finally, prion like aggregation and spreading ability of mutant SOD1 has been demonstrated in cultured cells (Grad et al., 2011) as well as seeding ability using spinal cord homogenate (Chia et al., 2010).

1.1.4 Chromosome 9 open reading frame 72 (*C9orf72*)

Repeated GGGGCC hexanucleotide expansion in the intronic portion of *C9orf72* has been recently identified as the most prevalent mutation among ALS patients, this pathological expansion can be found in ~40% of fALS, ~25% of familial FTD (fFTD), and in ~7% of apparent sALS subjects (DeJesus-Hernandez et al., 2011; Majounie et al., 2012; Renton et al., 2011). The hexanucleotide sequence is repeated up to 23 times in normal individuals, while it has been found repeated hundreds or thousands of times in affected patients. ALS patients with an expansion in *C9orf72* show a histology of cytoplasmic inclusions positive for ubiquitin and TDP-43 on neuropathological examination (DeJesus-Hernandez et al., 2011). Additionally TDP-43 is absent from inclusions in the cerebellar granular and molecular layers, and from the pyramidal cell layers of the hippocampus in patients with *C9orf72* expansion, suggesting that additional components other than TDP-43, such as ubiquilin-2 (*UBQLN2*) or sequestosome 1 (p62/SQSTM1), are present in the inclusions. The exact pathological mechanism for

C9orf72 hexanucleotide expansion is not yet understood, but 3 different mode have been hypothesized:

- a gain of RNA toxicity, by sequestration of RNA-binding proteins and other RNA species which would disrupt cellular metabolism (Renton et al., 2014; Xu et al., 2013)
- a gain of protein toxicity, due to the expression of a mutant protein containing repeated expansion
- a loss of function through haploinsufficiency, as reduced brain expression of a *C9orf72* protein isoform mRNA has been found (DeJesus-Hernandez et al., 2011; Renton et al., 2011)

The gain of RNA toxicity of *C9orf72* is suggested by intranuclear RNA foci found in ALS patients' with mutated *C9orf72* (DeJesus-Hernandez et al., 2010). These foci may trap one or more RNA-binding proteins, inhibiting their function especially in RNA processing. Regarding haploinsufficiency, a reduction of both isoforms of *C9orf72* mRNA levels has been demonstrated, however evidence of a reduction of the correspondent protein is still lacking. Little is known about *C9orf72* protein function, however bioinformatical analyses have predicted it should be a DENN (Differentially Expressed in Normal and Neoplastic cells) domain protein, that could act as a guanine nucleotide exchange factor for small GTPases. A recent study provided the first experimental evidence to confirm this: *C9orf72* was found to regulate endosomal trafficking and autophagy in neuronal cells and primary neurons (Farg et al., 2014; Levine et al., 2013; Zhang et al., 2012). This suggests that certain aspects of the ALS and FTD disease pathology might result from haploinsufficiency of *C9orf72*, leading to a defect in

intracellular membrane traffic, either exocytosis or endocytosis, in addition to the strong possibility of RNA-mediated toxicity.

1.1.5 Transactive response DNA binding 43-kDa (TDP-43)

TDP-43 is a 414 amino acid protein encoded by 6 exons; the gene contains 2 RNA recognition motifs (RRM 1 and 2) and a C-terminal glycine-rich region that is reputed to mediate protein-protein interactions, mainly with others hnRNPs (Buratti et al., 2005; Lagier-Tourenne et al., 2010). Mutations in the TARDBP gene, encoding for TDP-43, have the proposed mutational frequency of ~5% for fALS and 0.5-2% for sALS and almost all ALS-associated TDP-43 mutations are dominantly inherited and located in the glycine-rich region (Da Cruz and Cleveland, 2011; Sreedharan et al., 2008). TDP-43 was originally identified as a transcriptional repressor that binds to TAR DNA of the human immunodeficiency virus type 1 (HIV-1), and thus its name (Ou et al., 1995). Consistent with its role in transcription, TDP-43 was subsequently found in human brain and in several cell culture systems. TDP-43 was shown to be associated with euchromatin – actively transcribed genes – in nuclear DNA; its RRM2 was proposed to mediate binding to nuclear DNA (Ayala et al., 2008). In 2006, TDP-43 was identified as the main component of ubiquitinated cytoplasmic inclusions in ALS (Neumann et al., 2006). In aggregates, TDP-43 is hyperphosphorylated and cleaved to generate abnormal C-terminal fragments. Moreover, while in unaffected neurons TDP-43 localizes in the cell nucleus, it is absent from the nuclei of neurons with ubiquitinated inclusions, suggesting a nucleo-cytoplasmic redistribution of the protein. These observations lead to intense speculations on the pathogenic role of TDP- 43 in ALS: toxicity might be caused by aggregating TDP-43 being sequestered away from its normal nuclear function or, conversely, TDP-43

aggregates might have a toxic gain-of-function independent of the protein's physiological cellular activities (Lagier-Tourenne et al., 2010; Strong, 2010; Ticozzi et al., 2011). Although the specific functions of TDP-43 in neuronal cells remain to be evaluated, the protein has been demonstrated to play a role in several biological processes, including gene transcription, splicing regulation, transport and stabilization of mRNA molecules (Buratti and Baralle, 2008) [see below the paragraph 1.2.1 "The physiological and pathological role of TDP-43" pag. 15].

1.1.6 Fused in sarcoma and translocated in liposarcoma (FUS/TLS)

FUS is a 526 amino acid protein encoded by 15 exons; the gene contains an N-terminal domain enriched in glutamine, glycine, serine, and tyrosine residues (QGSY region), an RRM, an arginine-rich region, an arginine and glycine rich (RG rich) region, and a C-terminal zinc finger motif (Lanson and Pandey, 2012; Sreedharan et al., 2008). As with the TDP-43 gene, most pathogenic FUS mutations are clustered in the glycine-rich region (Da Cruz and Cleveland, 2011; Lagier-Tourenne et al., 2010; Lanson and Pandey, 2012). Similar to TDP-43-linked ALS, the inheritance pattern in FUS-associated ALS is autosomal dominant. Just as is the case with TDP-43, most FUS mutations are missense mutations, with only rare exceptions. Most patients develop a classical ALS phenotype, although occasional cases of ALS in young women with FUS mutations have been reported (Huang et al., 2010). Similar to TDP-43, FUS is chiefly nuclear in distribution in most cell types. Postmortem analysis of brain and spinal cord from ALS patients with FUS mutations has shown abnormal FUS-positive cytoplasmic inclusions in neurons and glial cells (Da Cruz and Cleveland, 2011; Kwiatkowski et al., 2009; Lagier-Tourenne et al., 2010; Vance et al., 2009). Interestingly, neuropathological examinations of tissues

from patients harboring FUS mutations showed an increased cytoplasmic FUS staining, FUS-immunoreactive dystrophic neurites, and cytoplasmic inclusions in lower motor neurons (Kwiatkowski et al., 2009; Vance et al., 2009). These mislocalized immunoreactive FUS inclusions are strikingly nonreactive for TDP-43, suggesting that neurodegenerative processes driven by FUS are independent of TDP-43 mislocalization. Regarding his molecular function, FUS is involved in several cellular pathways, including transcriptional regulation, maintenance of genomic stability, and splicing, nucleocytoplasmic shuttling, transport, and maturation of mRNAs (Law et al., 2006). Furthermore, in the central nervous system, the protein is involved in regulating mRNA transport towards the dendrites and synaptic plasticity upon the activation of glutamate receptors (Fujii et al., 2005). [see below the paragraph 1.2.2 “The physiological and pathological role of FUS” pag. 18].

1.2 The evidences for altered RNA metabolism in Amyotrophic Lateral Sclerosis (ALS)

The dynamic regulation of gene expression and its linkage to protein expression is increasingly seen to be governed by determinants of RNA metabolism that regulate the post-transcriptional processing of mRNA, including pre-mRNA splicing and processing, editing, transport, stabilization and translation, and degradation (Bolognani and Perrone-Bizzozero, 2008). TDP-43 and FUS, two ALS-related RNA binding proteins, have been documented to play a role in transcriptional regulation, RNA stability, microRNA (miRNA) processing and RNA splicing (Buratti and Baralle, 2008; Colombrita et al., 2012; Kawahara and Mieda-Sato, 2012; Morlando et al., 2012). Figure 1 summarizes the role of TDP-43 and FUS at different steps of RNA processing.

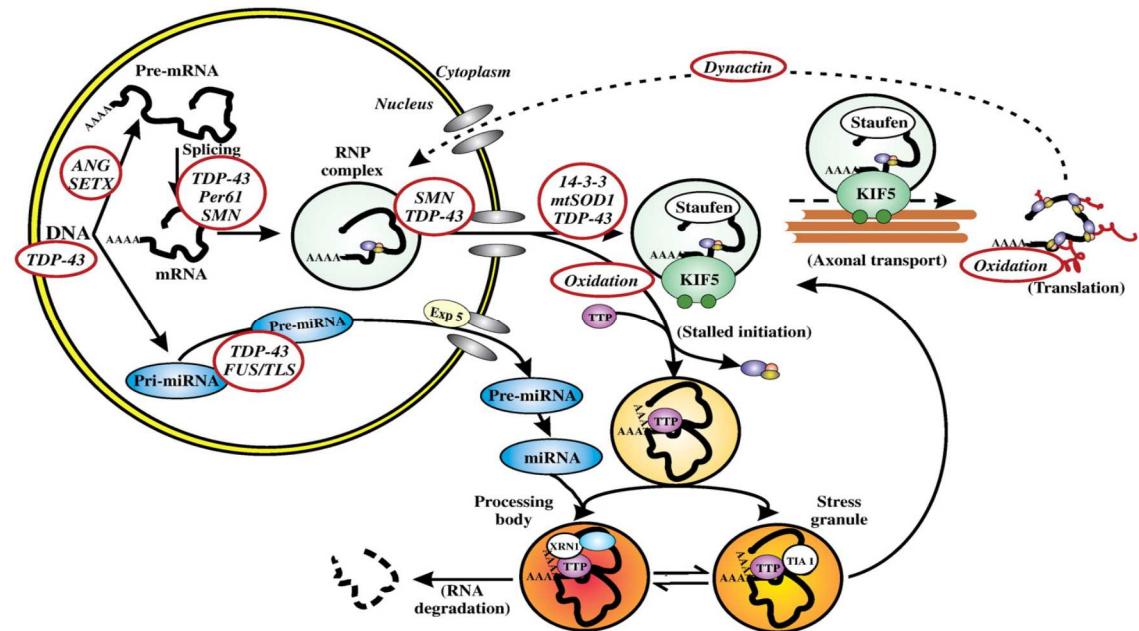


Figure 1. Schematic illustration of RNA metabolism and potential points of impact for ALS associated mutations or disease processes. Following gene transcription, pre-mRNA is modified by the spliceosome into mRNA which is then “bundled” in RNP complexes (RNA granules) and actively transported to the cytoplasm. In the mature neuron, RNA granules can exist in three forms, including transport granules in which the mRNA is translationally quiescent and transported to the sites of nascent protein synthesis. At these sites, an active polyribosome is reconstituted and translation proceeds. For many proteins, retrograde transport of either intact protein or degradation products allows for further regulation of gene expression. The RNA granule can also be modified in response to stalled initiation to give rise to either a stress granule, in which instance the mRNA is “held” until such time as needed, or a processing body (P-body) in which mRNA is degraded. The process of mRNA degradation within the P-body is governed in part by the specific association between the mRNA and miRNAs. The potential sites, or pathways, at which the interaction of RNA binding proteins or proteins associated with RNA processing, and which have been identified as disease modifying or causative agents in ALS, are indicated (Strong, 2010).

1.2.1 The physiological and pathological role of TDP-43

TDP-43 is a highly conserved protein ubiquitously expressed in many tissues, where it is present in neuronal and glial nuclei and to a lesser extent in the cytoplasm. It can shuttle between nucleus and cytosol, but at steady state it is mainly localized within the nucleus. TDP-43 has been shown to bind to more than 6000 pre-mRNA (Ayala et al., 2008; Buratti and Baralle, 2008; Buratti et al., 2004; Buratti et al., 2001; Polymenidou et al., 2011; Strong et al., 2007), affecting RNA maturation in several ways:

- it binds to more than 600 long intron containing mRNAs and sustains their levels (i.e. stabilize them);
- it binds to the splicing sites near and far from exon-intron junctions, influencing the splicing pattern of about 950 mRNAs;
- it is implied in stability and transport of more than 1000 transcripts by binding their 3' untranslated regions (UTR);
- it also possesses binding sites for noncoding RNAs (ncRNA) whose functions include chromatin remodeling, transcription regulation and post-transcriptional processing;
- it may exert further functions by binding small ncRNA and micro RNA (miRNA), a matter still unexplored.

Although TDP-43 is an ALS-related protein, the exact mechanism thought it exerts its toxic action is not known. A possible disease mechanism could be a gain of toxic function (Ayala et al., 2011), this has been proven by the fact that an increase (by less than a factor of 2) in its levels by loss of regulation of its production is highly deleterious (Igaz et al., 2011). TDP-43 levels are strictly regulated by an intrinsic autoregulatory pathway, as

inactivation of one copy of the gene in mice does not reduce the protein mRNA levels. Autoregulation is deemed to be mediated by TDP-43 dependent splicing of an intron in the 3' UTR of its own mRNA, and splicing of this gene leads to an unstable RNA which decays (Polymenidou et al., 2011). Furthermore, overexpression of wild-type human TDP-43 in neurons of flies, zebrafish embryos, and worms (*C. elegans*), also produces neurodegeneration accompanied by decreased locomotor activity, motor deficits, reduced lifespan, and motor neuron loss (Ash et al., 2010; Kabashi et al., 2010; Li et al., 2010). Not surprisingly, the severity of the motor phenotypes correlates with the level at which the transgene accumulates in neurons. Excess expression of wild-type human TDP-43 in the CNS of mice has also been shown to be toxic in a dose-dependent manner (Wils et al., 2010) confirming results previously obtained from other animal models. Two research groups have reported consequences of expressing ALS-linked mutants of TDP-43 in mice (Stallings et al., 2010; Wegorzewska et al., 2009). Accumulation of mutant human TDP-43 carrying the A315T mutation (TDP-43^{A315T}) to 3 to 4-fold above endogenous TDP-43 levels in the CNS caused loss of pyramidal neurons in cortical layer V, as well as of motor neurons in the spinal cord, with attendant gait abnormality and premature death, all features reminiscent of ALS. A key question, not yet clearly answered, is whether ALS-linked mutant TDP-43 triggers a far higher degree of toxicity than the elevated wild-type protein in mice. One recent study has directly attempted to address this question. Analysis of transgenic mice expressing human wild-type TDP-43 or TDP-43^{M337V} led these researchers to conclude that only human ALS-linked mutant TDP-43 produces motor dysfunction (Stallings et al., 2010). In summary, therefore, expression of human TDP-43 carrying an ALS-linked mutation appears to produce far greater toxicity than higher levels of wild-type TDP-43 in these models.

Another possible mechanism of disease is the loss of function due to TDP-43 nuclear depletion. TARDBP is a fundamental gene and its loss has been proven to be fatal in the embryonic stage of life, while the complete removal after birth is rapidly lethal without motor neuron disease (MND) (Chiang et al., 2010). Loss of TDP-43 function in Drosophila by gene deletion or its down-regulation by siRNA is deleterious in flies, leading to lethal (Huang et al., 2011) or semi-lethal (Li et al., 2010) phenotypes. The surviving null flies exhibit defects in locomotor behavior, reduced lifespan, reduced dendritic branching, and neuronal death. Similarly, knockdown of TDP-43 in zebrafish, using antisense oligonucleotides, causes a defective motor phenotype with motor axon degeneration but without clear lethality (Kabashi et al., 2010). The motor abnormalities in both flies and zebrafish can at least be partially rescued by wild-type TDP-43 (Kabashi et al., 2010). Loss of TDP-43 in mice following homozygous disruption of the TDP-43 gene results in lethality in embryos (Kraemer et al., 2010), suggesting that TDP-43 is essential for early embryogenesis in the mouse. Heterozygous TDP-43 gene disruption in mice has led to inconsistent findings, ranging from aged mice that are phenotypically indistinguishable from wild-type to those showing modest motor behavioral abnormalities (Kraemer et al., 2010). Animal models with selective inactivation of TARDBP genes produce MND with ALS pathology (Iguchi et al., 2013); however despite its contribute to disease progression, the sole loss of TDP-43 functions has been proved insufficient to cause a fatal MND.

TDP-43 is fundamental in stabilizing mRNAs with very long introns including those encoding FUS, as well as those of other ALS-related genes (ALSIN, CHMP2B, vasolin-containing protein (VCP), VAPB); some of the deregulated mRNAs are crucial for motor neuron survival, such as those encoding low molecular weight filaments (Strong et al.,

2007) and proteins implied in homeostasis of the distal part of the motor neuron axon (Strong et al., 2005). This may explain the molecular mechanism underlying the selective degeneration of motor neurons.

1.2.2 The physiological and pathological role of FUS

FUS is a member of the TET protein family, its C-terminal region has protein and RNA-binding capability while the N-terminal region appears to be involved in transcriptional activation. FUS C-terminal sequence motif binds to nuclear import receptor by which it usually localizes on the nucleus surface. There are 2 clusters of ALS/FTD-linked mutations: mutations of the low complexity prion like domain at N-terminal region and mutations in the C-terminal nuclear localization signal. C-terminal mutations have been shown to weaken the affinity of FUS to nuclear import receptor, resulting in cytoplasmic localization of mutant FUS (Zhang and Chook, 2012). Furthermore FUS is an intrinsically aggregation-prone protein, and it is possible that mutations at the C-terminal modulate the aggregation kinetics, increasing its propensity to aggregate. Similarly to TDP-43, FUS can bind single and double stranded DNA as well as RNA it affects both transcription and splicing.

- ❖ *Transcription.* Along with the other members of TET protein family, FUS has been shown to associate with RNA polymerase II and its transcription factor. By binding with several nuclear hormone receptors it can activate different promoters and influence transcription of specific genes (Tan et al., 2012).
- ❖ *Splicing.* FUS has been identified as part of the spliceosome machinery (Hartmuth et al., 2002), it has been demonstrated that its association with the spliceosome

influences the splicing pattern of more than 1000 RNAs (Lagier-Tourenne et al., 2012).

Moreover, FUS also possess important role in the cytosol. Indeed, FUS and TDP-43 tend to aggregate and form the RNA granules thanks to their prion like domains. RNA granules assembly is a physiological cell response when exposed to stressing conditions, under normal conditions both these proteins tend to reversibly bind together forming amyloid like fibers aggregates. Cytoplasmic bodies thus formed include: processing bodies (containing RNA decay machinery), stress granules (containing translation machinery) and transporting granules (containing RNA destined to local translation). [see below the paragraph 1.2.3 “Stress granules” pag. 20]. Furthermore, FUS has also been shown to affect dendritic spine formation in cultured hippocampal neurons, suggesting that FUS may play an important role in regulating synaptic function.

It is currently unclear whether loss of FUS, like as TDP-43, in the nucleus (loss of function) or its accumulation in cytoplasmic aggregates (toxic gain of function), or both, lead to the pathogenesis of ALS.

A loss of nuclear function of FUS protein is probably a component of the disease process, since nuclear clearing and cytoplasmic accumulation have been reported in surviving neurons of patients with mutations in FUS gene (Kwiatkowski et al., 2009). To date, a study in zebrafish has described the expression of wild-type or ALS-linked mutants of FUS (Bosco et al., 2010a). Unlike similar expression of wild-type or ALS-linked mutant TDP-43, no significant alterations in motor neuron morphology or motor axon outgrowth were found with either wild-type or mutant FUS. Transgenic rats conditionally expressing human wild-type and ALS-linked mutant FUS (FUS^{R521C}) revealed that expressing

mutant protein at levels 3- to 6-fold above endogenous levels in the CNS led to loss of cortical and hippocampal neurons, degeneration of motor axons, increased denervation of neuromuscular junctions and ultimately paralysis in 30 – 70 days after induction of the transgene (Huang et al., 2011). Altogether in these rat experiments, accumulation of mutant FUS in the CNS appears to be more toxic than wild-type protein.

A mechanism of gain of toxic function is less probable as no currently published mouse model stably expresses mutations of FUS leading to ALS.

1.2.3 Stress granules

In response to stressful conditions, eukaryotic cells require a rapid adaption for survival; they need to produce cytoprotective proteins and conserve energy. The formation of stress granules (SGs) in a series of reversible steps is crucial in the lattermost process. These granules facilitate cell survival by translational arrest of non-essential transcripts and by sequestering pro-apoptotic proteins and regulators of cell growth. SGs are cytoplasmic, non membrane enclosed particles containing different classes of components (reviewed by (Anderson and Kedersha, 2008). The first class consists of initiation complexes and includes mRNA transcripts, Translation Initiation Factor 3 (eIF3), Translation Initiation Factor 4B (eIF4B), Translation Initiation Factor 4F (eIF4F), Poly-A Binding Protein 1 (PABP-1) and small ribosomal subunits. The second class of components consist of RNA-binding proteins with some members of this group linked to translational silencing, others are linked to RNA decay and still others are RNA-binding proteins not related to RNA translation or decay. The third class of SG component interacts with core components through “piggyback” interactions. SGs are closely related to another class of RNA granules – processing bodies (P-bodies) (Parker and Sheth, 2007). SGs and P-bodies

are both cytoplasmic RNA-protein granules without a membrane (Souquere et al., 2009); both are assembled in response to stress (Raaben et al., 2007); growth in size depends on retrograde transport along microtubules (Loschi et al., 2009). They are assembled on untranslated mRNAs from dissembled polysomes (Cougot et al., 2004; Kedersha et al., 2000), and both share a large number of proteins (Figure 2). However, SGs and P-bodies differ in several ways:

- SGs are larger than P-bodies and appear as more irregular, looser and more granular structures that often contain regions of cytoplasm, while P-bodies are compact, dense structures (Souquere et al., 2009).
- SGs only emerge in response to stress (Kedersha et al., 2000), whereas a small number of P-bodies are observed in unstressed cells (van Dijk et al., 2002).
- SGs, but not P-bodies, usually require eIF2a phosphorylation for assembly upon stress (Kedersha et al., 2002).
- SGs are defined by translation initiation factors, including the non-canonical 48S pre-initiation complex (Kedersha et al., 2002; Kedersha et al., 1999), while P-bodies are defined by components of mRNA decay machinery (Kedersha et al., 2000; van Dijk et al., 2002).
- Although SGs and P-bodies can contain the same species of RNAs, SGs contain mRNAs that are polyadenylated (Kedersha et al., 2000; Kedersha et al., 1999) while in P-bodies, mRNA lack a poly(A)-tail (Anderson and Kedersha, 2008; Kedersha et al., 2002).

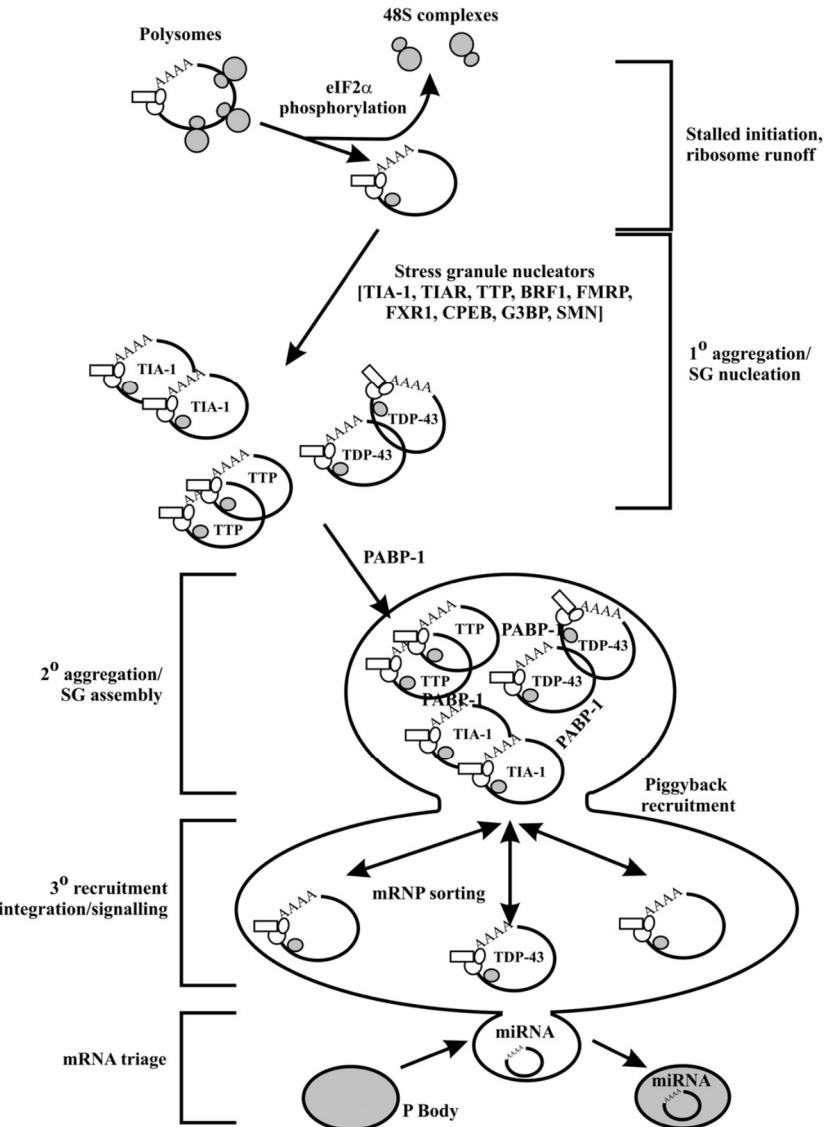


Figure 2. Schematic illustration of the 5 stages of stress granule (SG) formation. In response to cellular injury or stress, phosphorylation of eIF2 α results in abortive initiation complexes with stalled initiation and the conversion of polysomes into 48S ribosomes. Primary aggregation and SG nucleation is dependant on the presence of free 48S complexes and can be initiated by multiple proteins which then become part of the SG they nucleate. Secondary aggregation through protein:protein interactions results in the progressive fusion of SG to form larger aggregates. In the next step, proteins that lack mRNA binding properties are recruited which then integrate the SG with specific signaling pathways and provide the SG with an ability to integrate aspects of cellular metabolism with the translational response to stress. At this stage, RNA is triaged either into a translationally quiescent compartment which protects the RNA from degradation until translation is reinitiated, or into a pathway in which the RNA is destabilized and degraded in P-bodies. The interchange between SGs and P-bodies is dynamic with reversible exchange of mRNA, with the current view being that the determination of whether a mRNA is stabilized or degraded being in part dependant on the nature of the miRNA interaction with its respective MRE (Strong, 2010).

SGs and P-bodies are highly dynamic and highly connected structures (Kulkarni et al., 2010). Indeed, in mammalian cells SGs and P-bodies are formed independently, since abrogation of SGs does not hinder P-bodies assembly and vice versa (Kedersha et al., 2002). However, P-bodies intermittently and transiently dock with SGs and with vary proteins, affecting the duration of contact between granules (Kedersha et al., 2002). This raise the possibility that messenger ribonucleoprotein particles (mRNPs) may be exchanged between both granules.

TDP-43 and FUS are nuclear proteins that rapidly shuttle to the cytoplasm in response to stress and colocalize with SGs (Ayala et al., 2008). However, the role of TDP-43 and FUS in the formation of SGs is unclear. Some studies have suggested that TDP-43 overexpression itself induces SG de novo formation (Liu-Yesucevitz et al., 2010). However, others have shown that TDP-43 overexpression is not sufficient for SG formation; rather, an additional stress such as oxidative and osmotic stress or heat shock is required (Dewey et al., 2011; Meyerowitz et al., 2011). It has been shown that TDP-43 knockdown in HeLa cells decreases the number of cells containing SGs formed after oxidative stress, as well as the size of SGs (Aulas et al., 2012). However, it has also been reported that no change in SG formation is induced by TDP-43 knockdown (Liu-Yesucevitz et al., 2010). The same controversial results have been found in the case of TDP-43 mutants. The most comprehensive study on the functional role of TDP-43 in SGs and cellular stress response has been performed by McDonald et al. (2011). These authors showed that TDP-43 and its binding partner hnRNP A2 are components of SGs formed in response to oxidative stress. Endogenous and overexpressed FUS show a re-distribution from the nucleus to the cytoplasm in response to a oxidative and osmotic

stress, where it colocalizes to SGs (Blechingberg et al., 2012; Sama et al., 2013; Yamaguchi and Kitajo, 2012) in a manner reminiscent of TDP-43. However, FUS knockdown has no impact on the formation of SGs in response to oxidative stress, indicating that SG formation does not require endogenous FUS. The role for FUS mutants in SG formation is less clear, with conflicting reports. These observations highlight the controversial nature of determining the exact role of either TDP-43 and FUS in SG formation but, taken together, these preceding observations provide strong evidence suggesting that several ALS-related RNA binding proteins participate to some extent in the formation and/or function of SGs.

Under non pathological conditions, TDP-43 and FUS localize into SGs that assemble and disassemble in a fully reversible manner. However, under pathological conditions the normal nuclear-cytoplasmic shuttling of TDP-43 and FUS is interrupted and formation of insoluble cytoplasmic inclusions of these proteins is observed. Some evidence points to SGs as precursors of pathological inclusions. As already discussed, ALS-related RNA-binding proteins that form cytoplasmic aggregates, including TDP-43 and FUS, have been shown to associate with SGs. Moreover, it has been shown that endogenous TDP-43 localized to SGs can subsequently form ubiquitin-positive protein aggregates (Parker et al., 2012). ALS-linked pathological proteins, such as TDP-43 and FUS, have the tendency to form stable, insoluble protein aggregates. This is because they possess domains within the protein that are intrinsically disordered, capable of converting from a soluble form into a self-propagating aggregate-prone conformation, analogous to the domain described in yeast and called “prion-like domain”. These aggregation-prone proteins would have the capability to attract more aggregation-prone proteins or even

wild-type proteins, as has been described for mutant FUS attracting wild-type FUS to SGs after oxidative stress in human neuronal cells (Vance et al., 2013).

Finally, pathological aggregation could also be influenced by chronic stress caused by environmental factors such as heavy metals, exogenous and endogenous retroviruses, as well as oxidative stress associated with aging, which could even directly affect RNA species. This leads then to a complex scenario in which multiple sources of stress could contribute to the formation of insoluble aggregates from SGs, irreversibly interfering with the normal cell response to stress and, finally with normal neuronal function.

1.2.4 The role of TDP-43 and FUS in miRNA pathway

Several aspects of RNA metabolism have been extensively described as being altered in ALS. Besides alterations involving SGs (previously described), alterations in mRNA stability and transcription have also been described (Strong, 2010). Among many other structural and functional similarities, TDP-43 and FUS have the ability to play key roles in canonical miRNA biogenesis (Starega-Roslan et al., 2011) [see also the paragraph 1.3 “microRNAs” pag. 31]. This property is primarily achieved by their ability to bind nascent and precursor miRNA transcripts (Buratti et al., 2010; Kawahara and Mieda-Sato, 2012; Morlando et al., 2012). Both TDP-43 and FUS have been shown by mass spectrometry to bind Drosha, which mediates the first step in miRNA maturation (Kim et al., 2009). FUS is recruited to chromatin at site of miRNA transcription and binds to corresponding primary miRNAs (Morlando et al., 2012). TDP-43 also interacts with Dicer in the cytoplasm to further cleave the precursor miRNA (Kawahara and Mieda-Sato, 2012). Thus, TDP-43 can influence miRNA biogenesis at both a primary and precursor level in the nucleus and cytoplasm, while FUS only mediates a nuclear miRNA

maturation step. Finally, the biogenesis of miRNA is significantly reduced when TDP-43 or FUS levels are reduced (Buratti et al., 2010; Morlando et al., 2012), confirming the function of these two ALS-related RNA-binding proteins in miRNA processing. This new role described for TDP-43 and FUS is highly important for ALS since both proteins regulate the biogenesis of miRNA relevant for neuronal function, differentiation and synapse formation (Edbauer et al., 2010; Laneve et al., 2007; Li et al., 2013; Morlando et al., 2012; Packer et al., 2008; Pathania et al., 2012). Although non-canonical miRNA biogenesis pathways are now being increasingly documented, the role of TDP-43 and FUS in these pathway in not yet known. The participation of miRNAs in ALS has only recently been documented. The first clue about miRNAs involvement in ALS came from studies in mice models with Dicer ablation. Dicer enzyme is part of the miRNA biogenesis and maturation in vivo, indicating that global loss of miRNA biogenesis is detrimental for neuronal survival (Shin et al., 2009; Tao et al., 2011).

1.3 MicroRNAs

1.3.1 Introduction

MiRNAs are a novel class of small (18–25 nucleotides), non-coding RNA molecules predicted to post-transcriptionally regulate at least half the human transcriptome (Friedman et al., 2009). The discovery, and subsequent characterization, of miRNAs has revealed an intriguing additional level of gene regulation that is fundamental in a diverse range of pathways including development, differentiation and pathological processes. Each miRNA is estimated to regulate around 200 targets, and mRNA transcripts may be regulated by multiple miRNAs (Krek et al., 2005; Lewis et al., 2003; Lim et al., 2005). The miRNA biogenesis pathway is highly conserved, as are many miRNA sequences and their target binding sites, highlighting their importance across evolution (Berezikov et al., 2005; Friedman et al., 2009). MiRNA genes are encoded either in intergenic regions under control of their own promoter, within the introns of protein coding genes or are exonic, overlapping with coding regions and transcribed by the host promoter (Rodriguez et al., 2004). The majority of miRNAs in humans are transcribed independently and putative promoters for the most of these have been identified (Ozsolak et al., 2008; Zhou et al., 2007). Over 40% of human miRNAs are found in clusters that are co-transcribed as polycistronic transcriptional units (Griffiths-Jones et al., 2008; Lee et al., 2002). Many miRNAs are highly temporally and spatially regulated, either via transcription factors or epigenetic mechanisms including DNA methylation and histone modification (Chuang and Jones, 2007). Overall, the mechanisms that control miRNA expression are similar to those of protein-coding genes with a trend toward regulation by their target mRNAs and double-negative feedback loops (Carthew and Sontheimer, 2009).

1.3.2 Biogenesis of microRNAs

Canonical pathway

The bulk of miRNAs are generated via the typical, canonical pathway of miRNA biogenesis (Figure 3). MiRNA genes are transcribed by RNA polymerase II (pol II) to generate long primary transcripts (pri-miRNAs), which can be several kilo bases long. The pri-miRNAs are capped, spliced and polyadenylated. They may encode a single miRNA, clusters of distinct miRNAs, or a protein and can therefore also act as mRNA precursors (Carthew and Sontheimer, 2009). The next step also takes place in the nucleus and is orchestrated by the microprocessor complex. The principal components of this complex are the RNase III enzyme known as Drosha and its binding partner Di George syndrome critical region gene 8 (DGCR8), a double-stranded RNA-binding protein (Denli et al., 2004). Drosha digests pri-miRNAs to release hairpin structures called precursor miRNAs (pre-miRNAs), which are 60–70 nucleotides in length. Exportin-5 interacts directly with the pre-miRNAs to mediate their export into the cytoplasm, where a second RNase III enzyme named Dicer, cleaves the pre-miRNA to generate a double-stranded miRNA duplex of ~22 nucleotides. Following Dicer processing the miRNA duplex is rapidly unwound as it associates with Argonaute (Ago) proteins, one strand is retained to become the mature miRNA and is loaded into RNA-induced silencing complexes (RISCs) to participate in mRNA regulation. The complementary strand, which is found at lower concentrations within the cell and is sometimes called the * sequence, was believed to be non-functional and rapidly degraded. However, recent studies have demonstrated that several miRNA * sequences associate with different Ago protein complexes to also become active (Czech and Hannon, 2011).

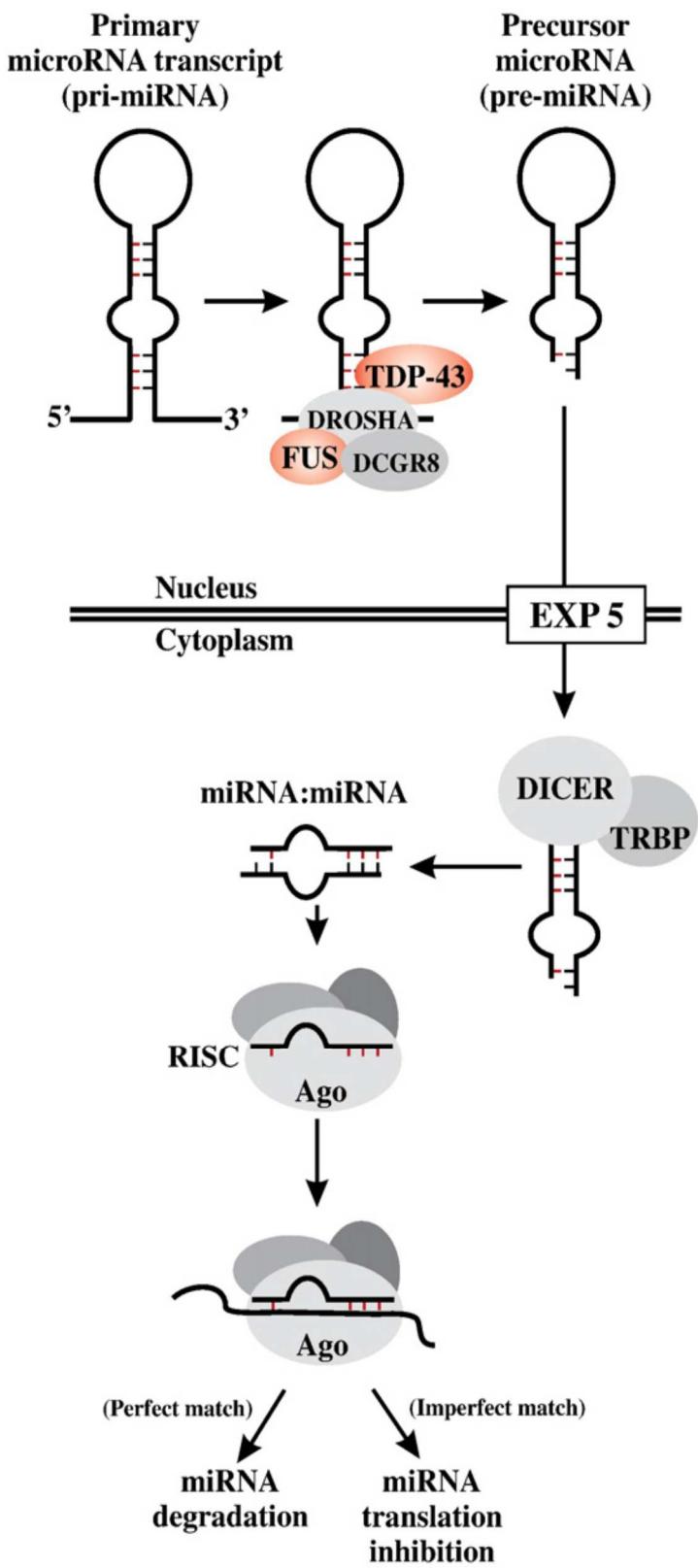


Figure 3. MiRNA biogenesis. The processing of miRNA includes both nuclear and cytoplasmic components. The primary miRNA transcript is predominantly transcribed by RNA polymerase II from intronic DNA, may be kilobases in length, and forms stem-loop structures that are cleaved by a microprocessor complex, the composition of which includes Drosha, TDP-43 and FUS/TLS (highlighted in red as fALS associated proteins). Following nuclear export, the resultant pre-miRNAs are cleaved by Dicer/TRBP to yield transient miRNA duplexes which are then associated with argonaute proteins (Argo), the catalytic component of the RNA-induced silencing complex (RISC), gives rise to the functionally mature miRNA. With Argo, the miRNA becomes associated with RNPs. If the miRNA has complete complementarity to its mRNA recognition element, the mRNA is directed to P-bodies for degradation. However, if the complementarity is incomplete, then translation inhibition is induced and the mRNA preferentially targeted to stress granules (Strong, 2010).

Non-canonical pathway

The advent of deep-sequencing technologies has led to the discovery of many miRNAs that are generated via alternative mechanisms, bypassing the usual Drosha/Dicer two-step processing (for in depth review see (Miyoshi et al., 2010)). In mammals four Drosha independent pathways have been identified, namely the mirtron pathway, small nucleolar RNA-derived, tRNA-derived and short hairpin RNA-derived pathways (Babiarz et al., 2008; Ender et al., 2008; Saraiya and Wang, 2008). The most common of these replaces the microprocessor step with a splicing event to produce short hairpin introns known as mirtrons that can be transported by Exportin-5 and cleaved by Dicer (Ruby et al., 2007). Mirtrons are relatively uncommon compared to canonical miRNAs, but have been identified throughout the animal kingdom and there is evidence to suggest a particular importance of mirtrons in the primate nervous system (Berezikov et al., 2007). In addition, there are two Dicer independent miRNA processing pathways. These are very rare with a single miRNA (miR-451) known to be produced via direct pre-miRNA loading onto Ago2 and miRNA-like small RNA sequences generated from tRNAs, with RNaseZ cleavage of pre-miRNAs in place of Dicer (Cheloufi et al., 2010; Haussecker et al., 2010; Lee et al., 2009).

1.3.3 miRNA mechanism of action

RISC is a generic term for a family of heterogeneous complexes containing Ago proteins that are involved with gene silencing (Pratt and MacRae, 2009). Once incorporated into the RISC, mature miRNAs act as a guide to direct target recognition via base-pairing interactions with mRNA transcripts, which are often located in the 3'UTR region (Bartel, 2009). The majority of animal miRNAs do not match their target sequences exactly,

however, nucleotides 2–6 of the miRNA are known as the “seed region” and are critical for target recognition (Lewis et al., 2005; Lewis et al., 2003). The extent of complementarity between a miRNA and its target mRNA sequence influences the downstream regulatory mechanism, with perfect matches leading to degradation, while mismatches result in translational repression. In humans the Ago2 protein catalyses target mRNA cleavage and subsequent degeneration of miRNA, although translational repression is the most prevalent mode of action for miRNAs in animals (Liu et al., 2004). The exact mechanism for repression remains unclear. There is evidence to support disruption of translation initiation, promotion of target mRNA deadenylation, sequestration of miRNAs and their targets to processing P-bodies and stress granules or RISC-mediated protein degradation after translation (Tang et al., 2008).

1.3.4 MicroRNAs and Amyotrophic Lateral Sclerosis

MiRNAs machinery has been found compromised in ALS. For instance the absence of processed miRNAs, due to Dicer deletion in spinal motor neurons, resulted in a mouse model with progressive paralysis, astrocytosis, and signs of axonopathy, classical features of ALS (Haramati et al., 2010). The authors identified a single miR-9-binding site on the neurofilament light polypeptide (NFLP) mRNA and observed that miR-9 was also downregulated in spinal muscular atrophy (SMA) models thereby suggesting direct evidence for miRNAs malfunction in motor neuron diseases. In another study, it was shown that the nuclear factor TDP-43, a major component of the inclusions in ALS, was found associated with Drosha complex, thereby involving miRNAs biogenesis. In TDP-43^{–/–} mice let-7b was downregulated, and miR-663 upregulated (Buratti et al., 2010). This phenomenon was correlated with the expression of FUS suggesting that this complex

acts as a cofactor involved in the biogenesis of a specific subset of miRNAs (Morlando et al., 2012). Several research groups acted as pioneers in the early characterization of miRNA involvement in ALS. Several studies have notably leveraged the SOD1-G93A mouse model for ALS (Gurney, 1994). A study performed in this model of familial ALS and subsequently validated in human ALS spinal cord tissues notably demonstrated strong expression of miR-155 (Koval et al., 2013). Furthermore, downregulation of miR-155 in ALS mice using oligonucleotide-based miRNA inhibitors or anti-miRs significantly prolonged survival. Profiling primary microglia cell cultures purified from the model also revealed a plethora of differentially expressed miRNAs including miR-22, miR-155, miR-125b, and miR-146b (Parisi et al., 2013). The group notably highlighted the miR-125b-based modulation of TNF α in ALS. A recent study using the SOD1-G93A model reported strong expression of miR-29 in ALS brain and spinal cord even though its knockdown did not lead to significant improvements in ALS-associated clinical endpoints (Nolan et al., 2014). Studies on human samples have also been conducted and have revealed the potential importance of miRNAs in ALS. First, a postmortem analysis of tissues isolated from the spinal cord at the lumbar level by the group of Michael Strong (Campos-Melo et al., 2013) revealed that the expression of numerous miRNAs was altered in ALS patients. Pathway analysis showed that these miRNAs were implicated in nervous system functions and cell death. The use of two prediction algorithms revealed three miRNAs (miR-146a*, miR-524-5p, and miR-582-3p) capable of interacting with the 3' UTR of the human low molecular weight neurofilament (NEFL) mRNA. A subsequent study from the same group revealed two additional miRNAs, miR-b1336 and miRb2403, capable of stabilizing NEFL transcripts in ventral lumbar spinal cord samples obtained from ALS patients (Ishtiaq et al., 2014). The presence of intraneuronal

neurofilamentous aggregates is a neuropathological hallmark of ALS, and reduced NEFL mRNA levels have been observed in degenerating spinal motor neurons. Motor neurons from the frontal cortex are also affected in ALS, and miRNA-related research to understand the role of these molecules in these cells is slowly emerging. Samples isolated from postmortem frontal cortex tissues of three ALS patients notably revealed an upregulation of miR-29a, miR-29b, and miR-338-3p (Shioya et al., 2010). However, due to a significant inter-individual variation, results were not subsequently validated by quantitative RT-PCR. Nevertheless, miR-338-3p upregulation in ALS patients has been also observed in blood leukocytes, CSF, serum, and spinal cord (De Felice et al., 2014). It is important to point out that skeletal muscle tissue represents another interesting source of potential biomarkers. Skeletal muscle mitochondrial dysfunction is believed to play a role in the progression and severity of ALS, and Russell et al. showed that miR-23a, miR-29b, miR-206, and miR-455 expressions were increased in skeletal muscle of ALS patients (Russell et al., 2013). MiR-206 is required for efficient regeneration of neuromuscular synapses after acute nerve injury, which probably accounts for its salutary effects in ALS (Lin and Friedlander, 2010). Evidences indicated that, although mice genetically lacking miR-206 were able to engage normal neuromuscular synapses during development, deficiency of miR-206 in the ALS mouse model accelerates the disease progression, at least in part through the histone deacetylase 4 (HDAC4) (Williams et al., 2009). HDAC4 is an important mediator neural activity action on muscle gene expression, and its expression is dramatically induced in muscles in response to denervation in ALS mice (Cohen et al., 2007). Remarkably, the phenotypes of miR-206 and HDAC4 mutant mice indicated that miR-206 and HDAC4 have opposite effects on retrograde signals required for the reinnervation. Bruneteau et al., investigated the role of the miRNA-206-

HDAC4 axis and showed that miR-206 was upregulated in ALS long-term survivors but it did not correlate with disease progression or reinnervation (Bruneteau et al., 2013). Clearly, several miRNAs seem to underlie the pathogenesis associated with ALS. It is only logical to wonder if any of those could potentially be leveraged as non-invasive circulating biomarkers to diagnose ALS and its various subsets.

1.3.5 MicroRNAs as appealing Biomarkers for ALS

Interestingly, living neurons and other CNS cells secrete miRNAs and other small non-coding RNAs into the extracellular space packaged in exosomes, microvesicles, or lipoprotein complexes. In addition, several studies have successfully isolated and quantified miRNAs from a variety of human body fluids including plasma or serum, urine, and saliva. Other factors positioning miRNAs as appealing biomarkers notably include their significant stability in body fluids as well as the relative ease of their detection given their well conserved sequences. These characteristics, coupled with the rapidly evolving improvements in technologies that allow for detection of RNA species from small amounts of biological material, have contributed to the strong interest dedicated towards the study of extracellular RNAs as potential biomarkers for neurodegenerative disorders. In a recent study, using the ALS mouse model SOD1-G93A, miR-206, involved in the maintenance of neuromuscular connectivity in ALS, was flagged as a potential circulating biomarker candidate as it exhibited strong upregulation in the serum of mice and ALS patients (Toivonen et al., 2014; Williams et al., 2009). MiR-206 upregulation was almost statistically significant in the presymptomatic stages of SOD1-G93A mice making it an interesting biomarker candidate for early diagnosis of ALS. This further reinforces the importance and challenges of identifying ALS-specific

circulating biomarkers to properly discriminate ALS from other CNS conditions. Recent work demonstrated that let-7 and miR-92 could notably differentiate ALS patients from patients diagnosed with relapsing-remitting multiple sclerosis, but not secondary progressive multiple sclerosis suggesting the latter possesses features present in other neurodegenerative diseases (Gandhi et al. 2013).

TDP-43 aggregates are observed in most ALS cases (Neumann et al., 2006) and identifying a biomarker associated with this target has been explored by several research teams. Freischmidt et al. (Freischmidt et al., 2013; Freischmidt et al., 2015) reported altered expression levels of five out of nine TDP-43-binding miRNAs in CSF and serum samples of sALS cases including miR-143-5p/3p. However, these authors found a poor correlation between CSF and serum levels of these miRNAs suggesting an independent regulation of TDP-43-binding microRNAs in the serum and CSF. Nonetheless, as proposed by these authors, these findings might be relevant for an easily accessible biological assessment of TDP-43 levels as well as of miRNAs regulating its expression. With the aim to find blood miRNAs specific to ALS and that correlates between CSF and serum, the group of De Felice first investigated the changes in miRNA expression profiles in leukocytes from ALS patients using a microarray strategy (De Felice et al., 2012). Several miRNAs were differentially expressed including miR-149, miR-328, miR-338-3p, miR-451, miR-583, miR-638, miR-665, and miR-1275. As mentioned previously, miR-338-3p overexpression was reported in frontal cortex tissues collected from three ALS patients (Shioya et al., 2010). Subsequent work was undertaken in a large cohort and showed an overexpression of miR-338-3p in blood leukocytes, CSF, serum, and spinal cord obtained from sALS patients (De Felice et al., 2014). MiR-338-3p expression was higher in ALS patients compared to healthy patients as well as to patients suffering from

other neurodegenerative disorders like PD, AD, and HD. Interestingly, miR-338-3p might relate with the higher glutamate levels observed in CSF of ALS patients described above. Indeed, one putative targets of deregulated miR-338p is the membrane-bound protein SLC1A2 which is the principal transporter that clears the excitatory neurotransmitter glutamate from the extracellular space at synapses in the CNS. Mutations and decreased expression of this protein are associated with certain forms of ALS (Rothstein et al., 1995).

Around the same time, the group of Weiner demonstrated that recruitment of inflammatory monocytes into the CNS played an important role in ALS progression (Butovsky et al., 2012). A thorough characterization of monocyte population was undertaken, and a unique miRNA signature within CD14+CD16– monocytes isolated from ALS patients with the SOD1 familial form was identified. This signature was similar to the one found in Ly6Chi monocytes from the mouse SOD1 model (Butovsky et al., 2012). MiRNAs such as miR-27a, miR-155, miR-142-5p, miR-223, and miR-532-3p were highly expressed in ALS patients compared to healthy controls or patients diagnosed with multiple sclerosis. MiR-27a could differentiate multiple sclerosis from ALS patients. Nonetheless, the same group showed that three miRNAs: miR-27b, miR-146a, and miR532-3p that were commonly elevated in CSF samples of ALS patients, in monocytes and microglia from SOD1 mice and from human ALS patients with the sporadic and familial forms (Butovsky et al., 2012). This finding highlights that a combination of miRNAs could represent a more plausible signature instead of only one miRNA.

1.4 Myocyte Enhancer Factor 2 (MEF2) proteins

1.4.1 Introduction

The myocyte enhancer factor 2 (MEF2) proteins belong to the MADS (MCM-1-agamous-deficiens-serum response factor) family of transcriptional regulators (Black and Olson, 1998). The MEF family of transcription factors includes four distinct vertebrate genes (*MEF2A-D*) encoding MEF2 forms. MEF2A was the first MEF transcription factor to be identified (Yu et al., 1992) and then three additional mammalian MEF2 iso-types (MEF2B, MEF2C, MEF2D) have subsequently been described (Shalizi and Bonni, 2005). These transcription factors were originally identified, as their family name implies, on the basis of their role in muscle differentiation. However, many tissues express the four members of the MEF2 family, which are found in distinct but overlapping patterns in neurons (Lyons et al., 1995). All four MEF2 proteins are expressed in the cerebral cortex and olfactory bulb. MEF2D transcripts are found throughout the developing central nervous system (CNS) through adulthood (Lyons et al., 1995), in contrast with the other MEF2 proteins showing more restricted patterns of expression. The expression pattern of MEF2C in the CNS is the most extensively characterized of the four MEF2 proteins; cortical expression of MEF2C protein is restricted to a subset of cortical neurons in layer II, IV and VI (McDermott et al., 1993). Interestingly, an alternative exon of MEF2C that extends the transactivation domain is expressed in the adult, but not in the developing cerebral cortex (Allen et al., 2002; McDermott et al., 1993). This alternative exon encodes a conserved phosphorylation site also present in MEF2A and MEF2D that negatively regulates MEF2 transcriptional activity (Gong et al., 2003). The reason for this developmental switch in MEF2C splicing has yet to be established, but it appears to render MEF2C sensitive to oxidative stress (Gong et al., 2003).

The MEF2 family is characterized by a MADS box and an immediately adjacent motif, namely MEF domain, which mediates DNA binding, dimerization and cofactors interactions (Black and Olson, 1998; McKinsey et al., 2002a). The MADS box is a highly conserved DNA-binding domain that targets proteins to A/T-rich sequences in gene regulatory regions (Figure 4).

MEF2 is a transcriptional activator that cooperates with other transcriptional factors to drive the expression of its target genes. A large collection of cofactors regulate the functions of MEF2 proteins by direct physical interaction with MEF2: GATA, GRIP1, histone deacetylases (HDACs), MyoD, NFAT, p300 and thyroid hormone receptor.

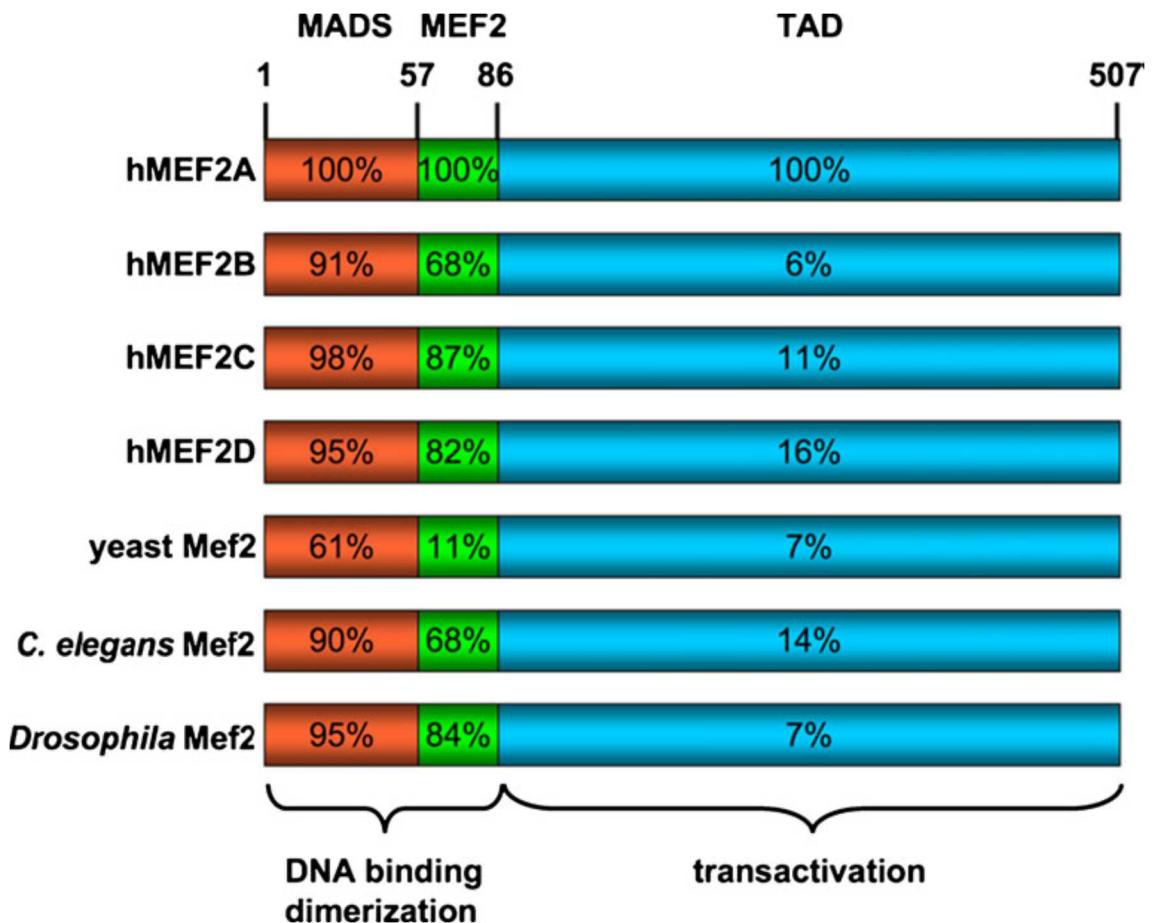


Figure 4. Sequence comparison of MEF2 (myocyte enhancer factor 2) transcription factors from various species (h human). The aminoacid numbering corresponds to the hMEF2A sequence and the sequence identities (in percentages) are related to hMEF2A (100%). The three domains, namely MADS (MCM-1-agamousdeficiens-serum response factor), MEF2 and TAD (transactivation domain) are highlighted in red, green and blue, respectively. (Potthoff and Olson, 2007).

1.4.2 Post-translational modifications and mechanisms of action of MEF2 transcription factors

MEF2 proteins serve as endpoints for multiple signaling pathways and thereby confer signal-responsiveness to downstream target genes (Figure 5). MAP kinase signaling pathways converge on MEF2 factors in organisms ranging from yeast to humans (Dodou and Treisman, 1997; Han et al., 1997; Kato et al., 1997). Phosphorylation of the transcription activation domain of MEF2 by MAP kinases augments its transcriptional activity, and the MAP kinase ERK5 serves as a MEF2 coactivator through its signal-dependent direct association with the MEF2 MADS domain (Yang et al., 1998). Indeed, MEF2 proteins are regulated by multiple phosphorylation events (McKinsey et al., 2002a; McKinsey et al., 2002b). The CDK5 and ERK5 can phosphorylate MEF2 proteins regulating directly MEF2 transcriptional activity (Gong et al., 2003; Kato et al., 2000). The p38 mitogen-activated protein (MAP) kinase (p38MAPK) also phosphorylates MEF2 in its transcriptional activation domain and thus stimulates its transcriptional activity (Zhao et al., 1999).

The small ubiquitin-like modifier (SUMO) is structurally related to ubiquitin and is covalently linked to lysine residues of proteins through an amide chemical bond. Sumoylation has now emerged as an important post-translational mechanism regulating the expression of transcription factors and cofactors. The MEF2 family is subject to sumoylation (Grégoire and Yang, 2005), indeed sumoylation of MEF2C at lysine 391 inhibits its transcriptional activity but does not block its DNA-binding activity. Of interest, the phosphorylation of serine 396 in MEF2C enhances its sumoylation *in vitro*.

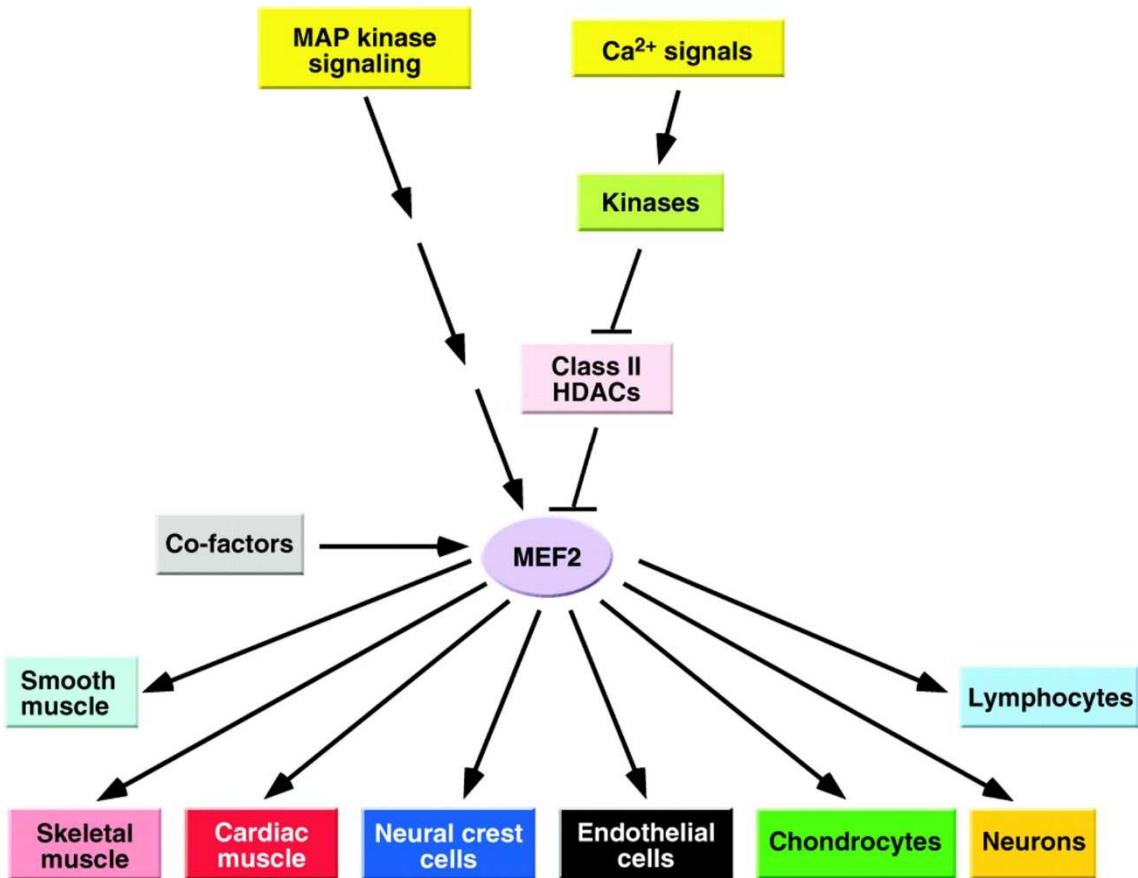


Figure 5. MEF2 as a central regulator of differentiation and signal responsiveness. MAP kinase signaling activates MEF2. Calcium dependent signals also activate MEF2 by stimulating calcium-dependent kinases that phosphorylate class II HDACs, thereby promoting their dissociation from MEF2 and derepressing MEF2 target genes. MEF2 recruits numerous co-factors to drive the differentiation of the various cell types shown. Although MAPK and HDAC signaling pathways have been implicated in the modulation of numerous MEF2-dependent developmental programs, these signaling pathways have not yet been shown to operate in all the cell types under MEF2 control.

Moreover, the serine 396A mutation has also been shown to reduce sumoylation of MEF2C *in vivo* and to enhance the transcription activity of MEF2C in reporter assays. In conclusion, these results demonstrate that phosphorylation of MEF2C at serine 396 facilitates its sumoylation with recruitment of co-repressors to inhibit transcription (Kang et al., 2006).

Calcium signaling pathways also modulate MEF2 activity through multiple mechanisms. In this regard, the activity of MEF2 is tightly governed by class IIa HDACs, which associate with the MADS domain and promote the formation of multiprotein repressive complexes on MEF2-dependent genes (McKinsey et al., 2001; McKinsey et al., 2002a; McKinsey et al., 2002b). Numerous calcium-regulated protein kinases, including protein kinase D (PKD) and calcium calmodulin-dependent protein kinases (CaMKs) phosphorylate class II HDACs on a series of conserved serine residues. This phosphorylation promotes the nuclear-to-cytoplasmic shuttling of these HDACs and the subsequent activation of MEF2 (McKinsey and Olson, 2005; Zhang et al., 2002). The regulated phosphorylation of class II HDACs thus provides a mechanism for the modulation of MEF2 target genes in response to physiological and pathological signaling.

1.4.3 MEF2 role in muscle differentiation

Vertebrate skeletal muscle differentiation is regulated by the cooperative interactions of myogenic transcription factors with MEF2, and by signaling pathways that regulate MEF2 activity. MEF2 factors alone do not possess myogenic activity but, in combination with bHLH transcription factors, drive and amplify the myogenic differentiation program (Molkentin et al., 1995; Wang et al., 2001). In addition to regulating numerous muscle structural genes, vertebrate MEF2 proteins regulate the expression of myogenic bHLH

genes, such as myogenin, as well as other genes that encode transcription factors, thereby providing a positive feed-forward loop that perpetuates and amplifies the decision to differentiate (Cheng et al., 1993; Molkentin and Olson, 1996). Moreover, MEF2C activates the expression of the class IIa HDAC, HDAC9, thereby creating a negative-feedback loop that modulates and restrains MEF2 from excessive activity (Haberland et al., 2007). This type of negative-feedback loop also confers signal responsiveness to MEF2-dependent gene programs through the regulated phosphorylation of class IIa HDACs. MEF2 establishes an additional level of myogenic regulation by regulating the expression of microRNAs, such as miR-1 and miR-133 (Sokol and Ambros, 2005; Zhao et al., 2005). Recently, several microRNAs were identified that affect skeletal muscle differentiation and proliferation. Interestingly, miR-1 has been shown to target class II HDACs (such as HDAC4) (Chen et al., 2006) to establish a positive feed-forward mechanism for MEF2 activation and skeletal muscle differentiation. This form of regulation, which enhances MEF2 activity, would oppose the direct activation of HDAC9 expression by MEF2, which represses MEF2 activity, illustrating the multifaceted mechanisms that exist to modulate MEF2. Presumably, these different regulatory loops are differentially controlled during various stages of skeletal muscle development and postnatal muscle remodeling. Despite extensive studies of MEF2 in skeletal muscle *in vitro*, relatively little is known about the roles of MEF2 proteins in vertebrate skeletal muscle *in vivo*. During mouse embryogenesis, *Mef2c* is the first *Mef2* gene to be expressed in the somite myotome (~E9.0), with *Mef2a* and *Mef2d* expressed about a day later (Edmondson et al., 1994). Global deletion of *Mef2a* or *Mef2d* has little or no effect on skeletal muscle development (Potthoff et al., 2007a; Potthoff et al., 2007b). Since *Mef2c*-null mice die around E9.5 (Lin et al., 1997), its role in skeletal muscle was not

examined until recently. Skeletal muscle deficient in *Mef2c* differentiates and forms myofibers during embryogenesis (Potthoff et al., 2007a; Potthoff et al., 2007b). However, on a C57BL/6 mixed genetic background, myofibers from mice with a skeletal muscle specific deletion of *Mef2c* rapidly deteriorate after birth owing to the occurrence of disorganized sarcomeres and to the loss of integrity of the sarcomere M-line (Potthoff et al., 2007a). Interestingly, similar results have been observed in zebrafish following the combined knockdown of *mef2c* and *mef2d* (Hinitz and Hughes, 2007). Notably, the muscle-specific overexpression of a super-active MEF2 protein in mice does not drive premature skeletal muscle differentiation (Potthoff et al., 2007b), consistent with previous in vitro studies that have demonstrated that MEF2 is not sufficient to drive skeletal muscle differentiation (Molkentin et al., 1995). These results reveal a key role for MEF2 proteins in the maintenance of sarcomere integrity and in the postnatal maturation of skeletal muscle.

1.4.4 MEF2 role in neuronal differentiation and survival

MEF2 proteins are highly enriched in neurons and exhibit distinct patterns of expression in different regions of the brain (Lyons et al., 1995), where they protect neurons from apoptotic death (Mao et al., 1999). Recently it was shown that MEF2 proteins regulate dendrite morphogenesis, differentiation of post-synaptic structures (Shalizi et al., 2006) and excitatory synapse number (Flavell et al., 2006). In addition, dephosphorylation of MEF2 by calcineurin regulates the expression of activity-regulated cytoskeletal-associated protein (*Arc*) and synaptic RAS GTPase-activating protein (*synGAP*; also known as *Syngap1*) (Flavell et al., 2006). ARC and synGAP play important roles in synaptic disassembly by promoting the internalization of glutamate receptors (Flavell et

al., 2006) and by inhibiting Ras-MAP signaling (Vazquez et al., 2004), respectively. In response to activity dependent calcium signaling, calcineurin dephosphorylates MEF2 at serine 408, signaling a switch from the sumoylation to the acetylation of its residue lysine 403. This change restricts the numbers of synapses that form (Flavell et al., 2006) and inhibits dendritic claw differentiation (Shalizi et al., 2006) through the activation of the orphan nuclear receptor *Nur77* and of *Arc* and *synGAP*. Thus, specific signaling events modulate gene expression by post-translationally modifying MEF2 to control synapse development and plasticity. The functions of individual MEF2 proteins and their roles in synaptic differentiation and disassembly *in vivo* have yet to be examined. Based on recent literature, however, MEF2 appears to play a role in synaptic plasticity, suggesting an important role for these proteins in learning and memory.

1.4.5 MEF2 role in T-cell development

The development and activation of thymocytes (T-cells) is a highly regulated process that requires multiple signaling cascades to direct changes in gene expression that alter T-cell state or fate. NUR77 is a crucial mediator of T-cell receptor (TCR)-induced apoptosis, and TCR-induced expression of NUR77 is mediated through two MEF2 sites in the *Nur77* promoter (Youn et al., 1999). In unstimulated T-cells, MEF2 is associated with transcriptional co-repressors, such as HDAC7 and Cabin1, which inhibit *Nur77* expression (Dequiedt et al., 2003). Following TCR activation, HDAC7 becomes dissociated from MEF2 through nucleus-cytoplasmic shuttling. Phosphorylation of HDAC7 by PKD1 recruits proteins 14-3-3 and translocates HDAC7 to the cytoplasm, allowing the activation of MEF2 (Parra et al., 2005). Conversely, HDAC7 is dephosphorylated by protein phosphatase 1 β (PP1 β ; also known as PPP1CB) and myosin

phosphatase targeting subunit 1 (MYPT1; also known as PPP1R12A), which are components of the myosin phosphatase complex that promote HDAC7 nuclear localization and repression of NUR77 expression (Parra et al., 2005). Therefore, regulation of MEF2 activity by association with transcriptional repressors is highly regulated in T-cells, and demonstrates the importance of MEF2-HDAC signaling in T-cell development, differentiation and thymocyte selection (Kasler and Verdin, 2007).

1.4.6 MEF2 and Neurotrophins

Neurotrophins (NTs) have profound effects on the development of the CNS and regulate differentiation, survival, and adaptive responses of neurons. Indeed, NTs protect many types of neurons from apoptosis both during development and in the adult (Levi-Montalcini and Booker, 1960). NTs including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT3, NT4/5, and NT6 bind to and activate specific receptor tyrosine kinases of the Trk family (Snider and Wright, 1996). Upon binding to and activating Trk, NTs can activate several intracellular signaling transduction systems including the phosphatidylinositol 3-kinase, protein kinase C, and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways (Segal and Greenberg, 1996).

BDNF is a neurotrophin involved in several aspects of neuronal and extra-neuronal life. It is synthesized as precursor protein, pro-BDNF, packed into secretory vesicles and released constitutively and upon stimulation. Cleavage by proteases, such as furin or proconvertases, produces mature neurotrophins. BDNF exerts its functions mainly at the synaptic cleft, through interaction with two types of receptor: the TrkB receptor and the p75 neurotrophin receptor ($p75^{NTR}$). In adult brains, mature BDNF predominantly interacts with TrkB inducing neuronal survival and synaptic plasticity. On the other hand,

pro-BDNF binds with higher affinity to p75^{NTR}, a member of the tumor necrosis factor superfamily of receptors, prompting apoptosis and a shrinkage of dendritic spines. Neurotrophins, and in particular way BDNF, are regulated by a wide range of different factors, including MEF2 proteins. Although the regulation of BDNF-MEF2 is very complex and only partially understood, recently some new evidences have emerged to clarify this mechanism. BDNF promote neuronal survival through stimulation of MEF2C, which has been largely attributed to its direct phosphorylation by extracellular signal-regulated kinases (ERK). Indeed, ERK1/2 and ERK5 are directly required for both the transcriptional and neuroprotective activity of MEF2 proteins in cortical neurons stimulated by BDNF (Cavanaugh et al., 2001; Liu et al., 2003; Shalizi et al., 2003; Wang et al., 2007). Moreover, Finsterwald and collaborators provided new details about BDNF-MEF2 regulation, directly involving the class II HDACs. They showed that BDNF induces the expression, phosphorylation and translocation of salt-inducible kinase 1 (SIK1) in cortical neurons through the ERK1/2 signaling pathway and that these effects were followed by the phosphorylation and nuclear export of HDAC5, leading to the activation of MEF2-mediated transcription (Finsterwald et al., 2013). However, this is not a one-way regulatory system and many evidences are present in the literature suggesting that MEF2D and MEF2C are not only induced by neurotrophins, but they are able to directly regulate BDNF expression (Flavell et al., 2008; Lyons et al., 2012; Pazyra-Murphy et al., 2009). In conclusion, the BDNF-MEF2 regulation system is a very complex molecular pathway not yet completely understood; but since BDNF and others neurotrophins have already been investigated in ALS [see below the paragraph 1.4.7 “Neurotrophins in ALS”], a possible role for MEF2 proteins in this neuromuscular disease could be hypothesized.

1.4.7 Neurotrophins in ALS

Various post mortem studies have been conducted in spinal cord and CNS of ALS patients in order to test the potential role of neurotrophins in motor neuron degeneration. ALS spinal motor neurons showed reduced neurotrophin-3 (NT-3) immunoreactivity compared to controls (Duberley et al., 1997) and changes in the receptors for BDNF were identified as well (Seeburger and Springer, 1993). Regarding the influence of neurotrophins on motor neuron growth and survival, in vitro studies showed BDNF to promote survival of motor neurons (Henderson et al., 1993). The same capability was demonstrated in vivo, in animal models of motor neuron death and peripheral nerve damage (Koliatsos et al., 1993; Meyer et al., 1992; Novikova et al., 1997; Sendtner et al., 1992; Yan et al., 1992) and of motor neuron degenerative disease (Ikeda et al., 1995; Mitsumoto et al., 1994; Sendtner et al., 1992). Animal studies also demonstrated the survival promoting properties of neurotrophic factors other than BDNF in motor neurons (Henderson et al., 1993; Sendtner et al., 1990). Taken together these observations emphasize the potential importance of neurotrophic factors in ALS and prompted clinical trials of systemically administered neurotrophic factors. Intrathecal infusion of BDNF was proved safe and feasible by a phase I/II clinical trial (Ochs et al., 2000), however a randomized, double-blind, placebo-controlled phase III study (1999) failed to demonstrate a statistically significant survival effect of BDNF in ALS.

1.5 Aim of the Study

Amyotrophic lateral sclerosis (ALS) is a fatal *neuro-degenerative* disease caused by the loss of both upper and lower motor neurons. Although the specific mechanisms underlying the pathogenesis of disease remain elusive, motor neuron death in ALS is the culmination of multiple aberrant biological processes, including derangements in cytoskeletal protein composition, mitochondrial dysfunction, impaired calcium and glutamate homeostasis, and enhanced oxidative injury. A major shift in our understanding of ALS pathogenesis occurred in 2006 with the identification of a 43-kDa transactive response (TAR) DNA-binding protein (TDP-43) as a key pathological substrate of cellular inclusions in sALS and non-SOD1 fALS (Neumann et al., 2006). It was soon followed in 2008 by the successful discovery of dominant mutations in the TDP-43 gene as a primary cause of ALS (Sreedharan et al., 2008), thus providing the proof of principle that aberrant TDP-43 can trigger neuronal degeneration and cause ALS. The identification of TDP-43 mutations was followed in tandem by the discovery of mutations in another RNA/DNA-binding protein, FUS in 2009, as a primary cause of fALS (Kwiatkowski et al., 2009). These findings have definitively confirmed that perturbations in the processing of RNA from gene transcription through degradation (RNA metabolism) may contribute to the motor neuron degeneration representing a key biological aspect of ALS pathogenesis.

In particular way, this study is focused on the role of two particular events of RNA metabolism.

The first one is concerning the involvement of microRNAs (miRNAs) in ALS. Since TDP-43 and FUS play a critical role in miRNAs biogenesis (Starega-Roslan et al., 2011),

these small non coding RNA molecules have been proposed to assume some important functions potentially involved in the pathogenesis of disease. Studies on human samples have been conducted and have revealed the potential importance of miRNAs in ALS. A postmortem analysis of tissues isolated from the spinal cord at the lumbar level by the group of Michael Strong (Campos-Melo et al., 2013) revealed that the expression of numerous miRNAs was altered in ALS patients and pathway analysis showed that these microRNAs were implicated in nervous system functions and cell death. Moreover, miRNAs alterations have also been found in leukocytes, serum and CSF (Butovsky et al., 2012; De Felice et al., 2014; De Felice et al., 2012; Freischmidt et al., 2013; Freischmidt et al., 2015) suggesting their potential usefulness as biomarkers. In this study we have investigated a panel of miRNAs in different SOD1(G93A) ALS models and in peripheral cells derived from sporadic ALS patients with the aim, not only to identify a potential their altered expression levels, but with the purpose to find a subgroup of miRNAs differentially expressed in a common manner in all the ALS models employed for the study. Since the sporadic and the familial form of ALS are clinically indistinguishable, they are characterized by very different histopathological hallmarks. For this reason, the opportunity to discover a possible subset of miRNAs differentially expressed in a common manner in different disease models, could let to identify a possible nodal event conserved in both these two different forms of disease. Furthermore, since miRNAs are stable and well dosable in blood and maintain a different expression profile in patients with respect to controls, they could be employed as disease biomarkers to facilitate diagnosis and follow up of disease.

The second part of the study concerns the assessment of a family of transcriptional factors in the ALS field. MEF2D and MEF2C are members of MEF2 family and play important

role both in muscle and in neurons. For this reason a possible their involvement in a *neuro-muscular* disease as ALS could be hypothesized. Recently, increased MEF2C mRNA levels have been shown in transgenic SOD1^{G93A} mice and similar results were observed after denervation in skeletal muscle cells obtained from wild type mice (Calvo et al., 2012), highlighting the importance of myogenic potential to compensate muscle damage induced by the neurodegenerative process. To date, no data exist about the expression and the function of MEF2D and MEF2C in ALS patients. Considering that the transcriptional activity of each tissue-specific MEF2 isoform on the responsive reporter elements is conserved in different cell lines (Sekiyama et al., 2012), we choose to investigate MEF2D and MEF2C gene and protein expression levels in peripheral blood mononuclear cells (PBMC), an easily accessible and widely used experimental tool able to recapitulate some typical pathological features previously found within CNS of ALS patients. MEF2 proteins could represent a novel potential pathological mechanism, still unexplored, inserted in the ambit of the transcriptional alterations in amyotrophic lateral sclerosis.

1.6 References

1999. A controlled trial of recombinant methionyl human BDNF in ALS: The BDNF Study Group (Phase III). *Neurology*. 52, 1427-33.
- Allen, M. P., et al., 2002. Adhesion-related kinase repression of gonadotropin-releasing hormone gene expression requires Rac activation of the extracellular signal-regulated kinase pathway. *J Biol Chem*. 277, 38133-40.
- Andersen, P. M., 2006. Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr Neurol Neurosci Rep*. 6, 37-46.
- Anderson, P., Kedersha, N., 2008. Stress granules: the Tao of RNA triage. *Trends Biochem Sci*. 33, 141-50.
- Ash, P. E., et al., 2010. Neurotoxic effects of TDP-43 overexpression in *C. elegans*. *Hum Mol Genet*. 19, 3206-18.
- Aulas, A., et al., 2012. Endogenous TDP-43, but not FUS, contributes to stress granule assembly via G3BP. *Mol Neurodegener*. 7, 54.
- Ayala, Y. M., et al., 2011. TDP-43 regulates its mRNA levels through a negative feedback loop. *EMBO J*. 30, 277-88.
- Ayala, Y. M., et al., 2008. Structural determinants of the cellular localization and shuttling of TDP-43. *J Cell Sci*. 121, 3778-85.
- Babiarz, J. E., et al., 2008. Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev*. 22, 2773-85.
- Bartel, D. P., 2009. MicroRNAs: target recognition and regulatory functions. *Cell*. 136, 215-33.
- Bendotti, C., et al., 2012. Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: implication for protein aggregation and immune response. *Prog Neurobiol*. 97, 101-26.
- Berezikov, E., et al., 2007. Mammalian mirtron genes. *Mol Cell*. 28, 328-36.
- Berezikov, E., et al., 2005. Phylogenetic shadowing and computational identification of human microRNA genes. *Cell*. 120, 21-4.
- Black, B. L., Olson, E. N., 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol*. 14, 167-96.
- Blechingberg, J., et al., 2012. Gene expression responses to FUS, EWS, and TAF15 reduction and stress granule sequestration analyses identifies FET-protein non-redundant functions. *PLoS One*. 7, e46251.
- Boillée, S., et al., 2006. Onset and progression in inherited ALS determined by motor neurons and microglia. *Science*. 312, 1389-92.
- Bolognani, F., Perrone-Bizzozero, N. I., 2008. RNA-protein interactions and control of mRNA stability in neurons. *J Neurosci Res*. 86, 481-9.
- Bosco, D. A., et al., 2010a. Mutant FUS proteins that cause amyotrophic lateral sclerosis incorporate into stress granules. *Hum Mol Genet*. 19, 4160-75.
- Bosco, D. A., et al., 2010b. Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. *Nat Neurosci*. 13, 1396-403.
- Bruneteau, G., et al., 2013. Muscle histone deacetylase 4 upregulation in amyotrophic lateral sclerosis: potential role in reinnervation ability and disease progression. *Brain*. 136, 2359-68.
- Buratti, E., Baralle, F. E., 2008. Multiple roles of TDP-43 in gene expression, splicing regulation, and human disease. *Front Biosci*. 13, 867-78.
- Buratti, E., et al., 2005. TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing. *J Biol Chem*. 280, 37572-84.

- Buratti, E., et al., 2004. Nuclear factor TDP-43 binds to the polymorphic TG repeats in CFTR intron 8 and causes skipping of exon 9: a functional link with disease penetrance. *Am J Hum Genet.* 74, 1322-5.
- Buratti, E., et al., 2010. Nuclear factor TDP-43 can affect selected microRNA levels. *FEBS J.* 277, 2268-81.
- Buratti, E., et al., 2001. Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping. *EMBO J.* 20, 1774-84.
- Butovsky, O., et al., 2012. Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. *J Clin Invest.* 122, 3063-87.
- Calvo, A. C., et al., 2012. Genetic biomarkers for ALS disease in transgenic SOD1(G93A) mice. *PLoS One.* 7, e32632.
- Campos-Melo, D., et al., 2013. Altered microRNA expression profile in Amyotrophic Lateral Sclerosis: a role in the regulation of NFL mRNA levels. *Mol Brain.* 6, 26.
- Carthew, R. W., Sontheimer, E. J., 2009. Origins and Mechanisms of miRNAs and siRNAs. *Cell.* 136, 642-55.
- Cavanaugh, J. E., et al., 2001. Differential regulation of mitogen-activated protein kinases ERK1/2 and ERK5 by neurotrophins, neuronal activity, and cAMP in neurons. *J Neurosci.* 21, 434-43.
- Cheloufi, S., et al., 2010. A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature.* 465, 584-9.
- Chen, J. F., et al., 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet.* 38, 228-33.
- Chen, Y. Z., et al., 2004. DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am J Hum Genet.* 74, 1128-35.
- Cheng, T. C., et al., 1993. Separable regulatory elements governing myogenin transcription in mouse embryogenesis. *Science.* 261, 215-8.
- Chia, R., et al., 2010. Superoxide dismutase 1 and tgSOD1 mouse spinal cord seed fibrils, suggesting a propagative cell death mechanism in amyotrophic lateral sclerosis. *PLoS One.* 5, e10627.
- Chiang, P. M., et al., 2010. Deletion of TDP-43 down-regulates Tbc1d1, a gene linked to obesity, and alters body fat metabolism. *Proc Natl Acad Sci U S A.* 107, 16320-4.
- Chuang, J. C., Jones, P. A., 2007. Epigenetics and microRNAs. *Pediatr Res.* 61, 24R-29R.
- Cohen, T. J., et al., 2007. The histone deacetylase HDAC4 connects neural activity to muscle transcriptional reprogramming. *J Biol Chem.* 282, 33752-9.
- Colombrita, C., et al., 2012. TDP-43 and FUS RNA-binding proteins bind distinct sets of cytoplasmic messenger RNAs and differently regulate their post-transcriptional fate in motoneuron-like cells. *J Biol Chem.* 287, 15635-47.
- Cougot, N., et al., 2004. Cytoplasmic foci are sites of mRNA decay in human cells. *J Cell Biol.* 165, 31-40.
- Couthouis, J., et al., 2011. A yeast functional screen predicts new candidate ALS disease genes. *Proc Natl Acad Sci U S A.* 108, 20881-90.
- Czech, B., Hannon, G. J., 2011. Small RNA sorting: matchmaking for Argonautes. *Nat Rev Genet.* 12, 19-31.
- Da Cruz, S., Cleveland, D. W., 2011. Understanding the role of TDP-43 and FUS/TLS in ALS and beyond. *Curr Opin Neurobiol.* 21, 904-19.
- De Felice, B., et al., 2014. miR-338-3p is over-expressed in blood, CFS, serum and spinal cord from sporadic amyotrophic lateral sclerosis patients. *Neurogenetics.* 15, 243-53.
- De Felice, B., et al., 2012. A miRNA signature in leukocytes from sporadic amyotrophic lateral sclerosis. *Gene.* 508, 35-40.
- DeJesus-Hernandez, M., et al., 2010. De novo truncating FUS gene mutation as a cause of sporadic amyotrophic lateral sclerosis. *Hum Mutat.* 31, E1377-89.
- DeJesus-Hernandez, M., et al., 2011. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron.* 72, 245-56.

- Deng, H. X., et al., 2011. Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. *Nature*. 477, 211-5.
- Denli, A. M., et al., 2004. Processing of primary microRNAs by the Microprocessor complex. *Nature*. 432, 231-5.
- Dequiedt, F., et al., 2003. HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. *Immunity*. 18, 687-98.
- Dewey, C. M., et al., 2011. TDP-43 is directed to stress granules by sorbitol, a novel physiological osmotic and oxidative stressor. *Mol Cell Biol*. 31, 1098-108.
- Dodou, E., Treisman, R., 1997. The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpkl mitogen-activated protein kinase pathway. *Mol Cell Biol*. 17, 1848-59.
- Duberley, R. M., et al., 1997. Neurotrophin-3-like immunoreactivity and Trk C expression in human spinal motoneurones in amyotrophic lateral sclerosis. *J Neurol Sci*. 148, 33-40.
- Edbauer, D., et al., 2010. Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. *Neuron*. 65, 373-84.
- Edmondson, D. G., et al., 1994. Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development*. 120, 1251-63.
- Ender, C., et al., 2008. A human snoRNA with microRNA-like functions. *Mol Cell*. 32, 519-28.
- Farg, M. A., et al., 2014. C9ORF72, implicated in amyotrophic lateral sclerosis and frontotemporal dementia, regulates endosomal trafficking. *Hum Mol Genet*. 23, 3579-95.
- Fecto, F., et al., 2011. SQSTM1 mutations in familial and sporadic amyotrophic lateral sclerosis. *Arch Neurol*. 68, 1440-6.
- Finsterwald, C., et al., 2013. Role of salt-inducible kinase 1 in the activation of MEF2-dependent transcription by BDNF. *PLoS One*. 8, e54545.
- Flavell, S. W., et al., 2006. Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. *Science*. 311, 1008-12.
- Flavell, S. W., et al., 2008. Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. *Neuron*. 60, 1022-38.
- Freischmidt, A., et al., 2013. Systemic dysregulation of TDP-43 binding microRNAs in amyotrophic lateral sclerosis. *Acta Neuropathol Commun*. 1, 42.
- Freischmidt, A., et al., 2015. Serum microRNAs in sporadic amyotrophic lateral sclerosis. *Neurobiol Aging*. 36, 2660.e15-20.
- Friedman, R. C., et al., 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*. 19, 92-105.
- Fujii, R., et al., 2005. The RNA binding protein TLS is translocated to dendritic spines by mGluR5 activation and regulates spine morphology. *Curr Biol*. 15, 587-93.
- Gong, X., et al., 2003. Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. *Neuron*. 38, 33-46.
- Grad, L. I., et al., 2011. Intermolecular transmission of superoxide dismutase 1 misfolding in living cells. *Proc Natl Acad Sci U S A*. 108, 16398-403.
- Greenway, M. J., et al., 2006. ANG mutations segregate with familial and 'sporadic' amyotrophic lateral sclerosis. *Nat Genet*. 38, 411-3.
- Griffiths-Jones, S., et al., 2008. miRBase: tools for microRNA genomics. *Nucleic Acids Res*. 36, D154-8.
- Grégoire, S., Yang, X. J., 2005. Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. *Mol Cell Biol*. 25, 2273-87.
- Gurney, M. E., 1994. Transgenic-mouse model of amyotrophic lateral sclerosis. *N Engl J Med*. 331, 1721-2.
- Haberland, M., et al., 2007. Regulation of HDAC9 gene expression by MEF2 establishes a negative-feedback loop in the transcriptional circuitry of muscle differentiation. *Mol Cell Biol*. 27, 518-25.

- Han, J., et al., 1997. Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature*. 386, 296-9.
- Haramati, S., et al., 2010. miRNA malfunction causes spinal motor neuron disease. *Proc Natl Acad Sci U S A*. 107, 13111-6.
- Hartmuth, K., et al., 2002. Protein composition of human prespliceosomes isolated by a tobramycin affinity-selection method. *Proc Natl Acad Sci U S A*. 99, 16719-24.
- Haussecker, D., et al., 2010. Human tRNA-derived small RNAs in the global regulation of RNA silencing. *RNA*. 16, 673-95.
- Henderson, C. E., et al., 1993. Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature*. 363, 266-70.
- Hinits, Y., Hughes, S. M., 2007. Mef2s are required for thick filament formation in nascent muscle fibres. *Development*. 134, 2511-9.
- Huang, C., et al., 2011. FUS transgenic rats develop the phenotypes of amyotrophic lateral sclerosis and frontotemporal lobar degeneration. *PLoS Genet*. 7, e1002011.
- Huang, E. J., et al., 2010. Extensive FUS-immunoreactive pathology in juvenile amyotrophic lateral sclerosis with basophilic inclusions. *Brain Pathol*. 20, 1069-76.
- Igaz, L. M., et al., 2011. Dysregulation of the ALS-associated gene TDP-43 leads to neuronal death and degeneration in mice. *J Clin Invest*. 121, 726-38.
- Iguchi, Y., et al., 2013. Loss of TDP-43 causes age-dependent progressive motor neuron degeneration. *Brain*. 136, 1371-82.
- Ikeda, K., et al., 1995. Effects of brain-derived neurotrophic factor on motor dysfunction in wobbler mouse motor neuron disease. *Ann Neurol*. 37, 505-11.
- Ishtiaq, M., et al., 2014. Analysis of novel NEFL mRNA targeting microRNAs in amyotrophic lateral sclerosis. *PLoS One*. 9, e85653.
- Kabashi, E., et al., 2010. Gain and loss of function of ALS-related mutations of TARDBP (TDP-43) cause motor deficits in vivo. *Hum Mol Genet*. 19, 671-83.
- Kabashi, E., et al., 2008. TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat Genet*. 40, 572-4.
- Kang, J., et al., 2006. Phosphorylation-facilitated sumoylation of MEF2C negatively regulates its transcriptional activity. *BMC Biochem*. 7, 5.
- Kasler, H. G., Verdin, E., 2007. Histone deacetylase 7 functions as a key regulator of genes involved in both positive and negative selection of thymocytes. *Mol Cell Biol*. 27, 5184-200.
- Kato, Y., et al., 1997. BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J*. 16, 7054-66.
- Kato, Y., et al., 2000. Big mitogen-activated kinase regulates multiple members of the MEF2 protein family. *J Biol Chem*. 275, 18534-40.
- Kawahara, Y., Mieda-Sato, A., 2012. TDP-43 promotes microRNA biogenesis as a component of the Drosha and Dicer complexes. *Proc Natl Acad Sci U S A*. 109, 3347-52.
- Kedersha, N., et al., 2002. Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules. *Mol Biol Cell*. 13, 195-210.
- Kedersha, N., et al., 2000. Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. *J Cell Biol*. 151, 1257-68.
- Kedersha, N. L., et al., 1999. RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. *J Cell Biol*. 147, 1431-42.
- Kim, V. N., et al., 2009. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. 10, 126-39.
- Koliatsos, V. E., et al., 1993. Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. *Neuron*. 10, 359-67.
- Koval, E. D., et al., 2013. Method for widespread microRNA-155 inhibition prolongs survival in ALS-model mice. *Hum Mol Genet*. 22, 4127-35.

- Kraemer, B. C., et al., 2010. Loss of murine TDP-43 disrupts motor function and plays an essential role in embryogenesis. *Acta Neuropathol.* 119, 409-19.
- Krek, A., et al., 2005. Combinatorial microRNA target predictions. *Nat Genet.* 37, 495-500.
- Kulkarni, M., et al., 2010. On track with P-bodies. *Biochem Soc Trans.* 38, 242-51.
- Kwiatkowski, T. J., et al., 2009. Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science.* 323, 1205-8.
- Lagier-Tourenne, C., et al., 2010. TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. *Hum Mol Genet.* 19, R46-64.
- Lagier-Tourenne, C., et al., 2012. Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs. *Nat Neurosci.* 15, 1488-97.
- Laneve, P., et al., 2007. The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells. *Proc Natl Acad Sci U S A.* 104, 7957-62.
- Lanson, N. A., Pandey, U. B., 2012. FUS-related proteinopathies: lessons from animal models. *Brain Res.* 1462, 44-60.
- Law, W. J., et al., 2006. TLS, EWS and TAF15: a model for transcriptional integration of gene expression. *Brief Funct Genomic Proteomic.* 5, 8-14.
- Leblond, C. S., et al., 2014. Dissection of genetic factors associated with amyotrophic lateral sclerosis. *Exp Neurol.* 262 Pt B, 91-101.
- Lee, Y., et al., 2002. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J.* 21, 4663-70.
- Lee, Y. S., et al., 2009. A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). *Genes Dev.* 23, 2639-49.
- Lefebvre, S., et al., 1995. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell.* 80, 155-65.
- Levi-Montalcini, R., Booker, B., 1960. DESTRUCTION OF THE SYMPATHETIC GANGLIA IN MAMMALS BY AN ANTISERUM TO A NERVE-GROWTH PROTEIN. *Proc Natl Acad Sci U S A.* 46, 384-91.
- Levine, T. P., et al., 2013. The product of C9orf72, a gene strongly implicated in neurodegeneration, is structurally related to DENN Rab-GEFs. *Bioinformatics.* 29, 499-503.
- Lewis, B. P., et al., 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* 120, 15-20.
- Lewis, B. P., et al., 2003. Prediction of mammalian microRNA targets. *Cell.* 115, 787-98.
- Li, Y., et al., 2010. A Drosophila model for TDP-43 proteinopathy. *Proc Natl Acad Sci U S A.* 107, 3169-74.
- Li, Z., et al., 2013. The FTD/ALS-associated RNA-binding protein TDP-43 regulates the robustness of neuronal specification through microRNA-9a in Drosophila. *Hum Mol Genet.* 22, 218-25.
- Lim, L. P., et al., 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature.* 433, 769-73.
- Lin, N., Friedlander, R. M., 2010. Regeneration of neuromuscular synapses: action of microRNA-206. *Neurosurgery.* 66, N19-20.
- Lin, Q., et al., 1997. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science.* 276, 1404-7.
- Liu, J., et al., 2004. Argonaute2 is the catalytic engine of mammalian RNAi. *Science.* 305, 1437-41.
- Liu, L., et al., 2003. ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. *Proc Natl Acad Sci U S A.* 100, 8532-7.
- Liu-Yesucevitz, L., et al., 2010. Tar DNA binding protein-43 (TDP-43) associates with stress granules: analysis of cultured cells and pathological brain tissue. *PLoS One.* 5, e13250.

- Loschi, M., et al., 2009. Dynein and kinesin regulate stress-granule and P-body dynamics. *J Cell Sci.* 122, 3973-82.
- Lyons, G. E., et al., 1995. Expression of *mef2* genes in the mouse central nervous system suggests a role in neuronal maturation. *J Neurosci.* 15, 5727-38.
- Lyons, M. R., et al., 2012. Members of the myocyte enhancer factor 2 transcription factor family differentially regulate *Bdnf* transcription in response to neuronal depolarization. *J Neurosci.* 32, 12780-5.
- Mackenzie, I. R., et al., 2010. TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. *Lancet Neurol.* 9, 995-1007.
- Majounie, E., et al., 2012. Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. *Lancet Neurol.* 11, 323-30.
- Malpass, K., 2012. Motor neuron disease: Functional screening identifies novel candidate risk genes in amyotrophic lateral sclerosis. *Nat Rev Neurol.* 8, 1.
- Mao, Z., et al., 1999. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. *Science.* 286, 785-90.
- Maruyama, H., et al., 2010. Mutations of optineurin in amyotrophic lateral sclerosis. *Nature.* 465, 223-6.
- McCord, J. M., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem.* 244, 6049-55.
- McDermott, J. C., et al., 1993. hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors. *Mol Cell Biol.* 13, 2564-77.
- McKinsey, T. A., Olson, E. N., 2005. Toward transcriptional therapies for the failing heart: chemical screens to modulate genes. *J Clin Invest.* 115, 538-46.
- McKinsey, T. A., et al., 2001. Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev.* 11, 497-504.
- McKinsey, T. A., et al., 2002a. MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends Biochem Sci.* 27, 40-7.
- McKinsey, T. A., et al., 2002b. Signaling chromatin to make muscle. *Curr Opin Cell Biol.* 14, 763-72.
- Meyer, M., et al., 1992. Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. *J Cell Biol.* 119, 45-54.
- Meyerowitz, J., et al., 2011. C-Jun N-terminal kinase controls TDP-43 accumulation in stress granules induced by oxidative stress. *Mol Neurodegener.* 6, 57.
- Mitsumoto, H., et al., 1994. The effects of ciliary neurotrophic factor on motor dysfunction in wobbler mouse motor neuron disease. *Ann Neurol.* 36, 142-8.
- Miyoshi, K., et al., 2010. Many ways to generate microRNA-like small RNAs: non-canonical pathways for microRNA production. *Mol Genet Genomics.* 284, 95-103.
- Molkentin, J. D., et al., 1995. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. *Cell.* 83, 1125-36.
- Molkentin, J. D., Olson, E. N., 1996. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. *Proc Natl Acad Sci U S A.* 93, 9366-73.
- Morlando, M., et al., 2012. FUS stimulates microRNA biogenesis by facilitating co-transcriptional Drosha recruitment. *EMBO J.* 31, 4502-10.
- Neumann, M., et al., 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science.* 314, 130-3.
- Nolan, K., et al., 2014. Increased expression of microRNA-29a in ALS mice: functional analysis of its inhibition. *J Mol Neurosci.* 53, 231-41.
- Novikova, L., et al., 1997. Effects of neurotransplants and BDNF on the survival and regeneration of injured adult spinal motoneurons. *Eur J Neurosci.* 9, 2774-7.

- Ochs, G., et al., 2000. A phase I/II trial of recombinant methionyl human brain derived neurotrophic factor administered by intrathecal infusion to patients with amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 1, 201-6.
- Ou, S. H., et al., 1995. Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. *J Virol.* 69, 3584-96.
- Ozsolak, F., et al., 2008. Chromatin structure analyses identify miRNA promoters. *Genes Dev.* 22, 3172-83.
- Packer, A. N., et al., 2008. The bifunctional microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in Huntington's disease. *J Neurosci.* 28, 14341-6.
- Parisi, C., et al., 2013. Dysregulated microRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation. *Cell Death Dis.* 4, e959.
- Parker, R., Sheth, U., 2007. P bodies and the control of mRNA translation and degradation. *Mol Cell.* 25, 635-46.
- Parker, S. J., et al., 2012. Endogenous TDP-43 localized to stress granules can subsequently form protein aggregates. *Neurochem Int.* 60, 415-24.
- Parra, M., et al., 2005. Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation. *J Biol Chem.* 280, 13762-70.
- Pathania, M., et al., 2012. miR-132 enhances dendritic morphogenesis, spine density, synaptic integration, and survival of newborn olfactory bulb neurons. *PLoS One.* 7, e38174.
- Pazyra-Murphy, M. F., et al., 2009. A retrograde neuronal survival response: target-derived neurotrophins regulate MEF2D and bcl-w. *J Neurosci.* 29, 6700-9.
- Polymenidou, M., et al., 2011. Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. *Nat Neurosci.* 14, 459-68.
- Potthoff, M. J., et al., 2007a. Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c. *Mol Cell Biol.* 27, 8143-51.
- Potthoff, M. J., Olson, E. N., 2007. MEF2: a central regulator of diverse developmental programs. *Development.* 134, 4131-40.
- Potthoff, M. J., et al., 2007b. Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. *J Clin Invest.* 117, 2459-67.
- Pratt, A. J., MacRae, I. J., 2009. The RNA-induced silencing complex: a versatile gene-silencing machine. *J Biol Chem.* 284, 17897-901.
- Raaben, M., et al., 2007. Mouse hepatitis coronavirus replication induces host translational shutoff and mRNA decay, with concomitant formation of stress granules and processing bodies. *Cell Microbiol.* 9, 2218-29.
- Renton, A. E., et al., 2014. State of play in amyotrophic lateral sclerosis genetics. *Nat Neurosci.* 17, 17-23.
- Renton, A. E., et al., 2011. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron.* 72, 257-68.
- Robberecht, W., Philips, T., 2013. The changing scene of amyotrophic lateral sclerosis. *Nat Rev Neurosci.* 14, 248-64.
- Rodriguez, A., et al., 2004. Identification of mammalian microRNA host genes and transcription units. *Genome Res.* 14, 1902-10.
- Rosen, D. R., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature.* 362, 59-62.
- Rothstein, J. D., et al., 1995. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann Neurol.* 38, 73-84.
- Ruby, J. G., et al., 2007. Intronic microRNA precursors that bypass Drosha processing. *Nature.* 448, 83-6.
- Russell, A. P., et al., 2013. Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis. *Neurobiol Dis.* 49, 107-17.
- Sama, R. R., et al., 2013. FUS/TLS assembles into stress granules and is a prosurvival factor during hyperosmolar stress. *J Cell Physiol.* 228, 2222-31.

- Saraiya, A. A., Wang, C. C., 2008. snoRNA, a novel precursor of microRNA in Giardia lamblia. *PLoS Pathog.* 4, e1000224.
- Seeburger, J. L., Springer, J. E., 1993. Experimental rationale for the therapeutic use of neurotrophins in amyotrophic lateral sclerosis. *Exp Neurol.* 124, 64-72.
- Segal, R. A., Greenberg, M. E., 1996. Intracellular signaling pathways activated by neurotrophic factors. *Annu Rev Neurosci.* 19, 463-89.
- Sekiyama, Y., et al., 2012. Functional gene expression analysis of tissue-specific isoforms of Mef2c. *Cell Mol Neurobiol.* 32, 129-39.
- Sendtner, M., et al., 1992. Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature.* 360, 757-9.
- Sendtner, M., et al., 1990. Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature.* 345, 440-1.
- Shalizi, A., et al., 2006. A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. *Science.* 311, 1012-7.
- Shalizi, A., et al., 2003. Characterization of a neurotrophin signaling mechanism that mediates neuron survival in a temporally specific pattern. *J Neurosci.* 23, 7326-36.
- Shalizi, A. K., Bonni, A., 2005. brawn for brains: the role of MEF2 proteins in the developing nervous system. *Curr Top Dev Biol.* 69, 239-66.
- Shin, D., et al., 2009. Dicer ablation in oligodendrocytes provokes neuronal impairment in mice. *Ann Neurol.* 66, 843-57.
- Shioya, M., et al., 2010. Aberrant microRNA expression in the brains of neurodegenerative diseases: miR-29a decreased in Alzheimer disease brains targets neurone navigator 3. *Neuropathol Appl Neurobiol.* 36, 320-30.
- Snider, W. D., Wright, D. E., 1996. Neurotrophins cause a new sensation. *Neuron.* 16, 229-32.
- Sokol, N. S., Ambros, V., 2005. Mesodermally expressed Drosophila microRNA-1 is regulated by Twist and is required in muscles during larval growth. *Genes Dev.* 19, 2343-54.
- Souquere, S., et al., 2009. Unravelling the ultrastructure of stress granules and associated P-bodies in human cells. *J Cell Sci.* 122, 3619-26.
- Sreedharan, J., et al., 2008. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science.* 319, 1668-72.
- Stallings, N. R., et al., 2010. Progressive motor weakness in transgenic mice expressing human TDP-43. *Neurobiol Dis.* 40, 404-14.
- Starega-Roslan, J., et al., 2011. The role of the precursor structure in the biogenesis of microRNA. *Cell Mol Life Sci.* 68, 2859-71.
- Strong, M. J., 2010. The evidence for altered RNA metabolism in amyotrophic lateral sclerosis (ALS). *J Neurol Sci.* 288, 1-12.
- Strong, M. J., et al., 2005. The pathobiology of amyotrophic lateral sclerosis: a proteinopathy? *J Neuropathol Exp Neurol.* 64, 649-64.
- Strong, M. J., et al., 2007. TDP43 is a human low molecular weight neurofilament (hNFL) mRNA-binding protein. *Mol Cell Neurosci.* 35, 320-7.
- Tan, A. Y., et al., 2012. TLS/FUS (translocated in liposarcoma/fused in sarcoma) regulates target gene transcription via single-stranded DNA response elements. *Proc Natl Acad Sci U S A.* 109, 6030-5.
- Tang, G., et al., 2008. The art of microRNA: various strategies leading to gene silencing via an ancient pathway. *Biochim Biophys Acta.* 1779, 655-62.
- Tao, J., et al., 2011. Deletion of astroglial Dicer causes non-cell-autonomous neuronal dysfunction and degeneration. *J Neurosci.* 31, 8306-19.
- Ticozzi, N., et al., 2011. Genetics of familial Amyotrophic lateral sclerosis. *Arch Ital Biol.* 149, 65-82.
- Toivonen, J. M., et al., 2014. MicroRNA-206: a potential circulating biomarker candidate for amyotrophic lateral sclerosis. *PLoS One.* 9, e89065.
- van Dijk, E., et al., 2002. Human Dcp2: a catalytically active mRNA decapping enzyme located in specific cytoplasmic structures. *EMBO J.* 21, 6915-24.

- Vance, C., et al., 2009. Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science*. 323, 1208-11.
- Vance, C., et al., 2013. ALS mutant FUS disrupts nuclear localization and sequesters wild-type FUS within cytoplasmic stress granules. *Hum Mol Genet*. 22, 2676-88.
- Vazquez, L. E., et al., 2004. SynGAP regulates spine formation. *J Neurosci*. 24, 8862-72.
- Wang, D. Z., et al., 2001. The Mef2c gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development. *Development*. 128, 4623-33.
- Wang, Y., et al., 2007. Brain-derived neurotrophic factor stimulates the transcriptional and neuroprotective activity of myocyte-enhancer factor 2C through an ERK1/2-RSK2 signaling cascade. *J Neurochem*. 102, 957-66.
- Wegorzewska, I., et al., 2009. TDP-43 mutant transgenic mice develop features of ALS and frontotemporal lobar degeneration. *Proc Natl Acad Sci U S A*. 106, 18809-14.
- Williams, A. H., et al., 2009. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science*. 326, 1549-54.
- Wils, H., et al., 2010. TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration. *Proc Natl Acad Sci U S A*. 107, 3858-63.
- Xu, Z., et al., 2013. Expanded GGGGCC repeat RNA associated with amyotrophic lateral sclerosis and frontotemporal dementia causes neurodegeneration. *Proc Natl Acad Sci U S A*. 110, 7778-83.
- Yamaguchi, A., Kitajo, K., 2012. The effect of PRMT1-mediated arginine methylation on the subcellular localization, stress granules, and detergent-insoluble aggregates of FUS/TLS. *PLoS One*. 7, e49267.
- Yamanaka, K., et al., 2008. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci*. 11, 251-3.
- Yan, Q., et al., 1992. Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature*. 360, 753-5.
- Yang, C. C., et al., 1998. Interaction of myocyte enhancer factor 2 (MEF2) with a mitogen-activated protein kinase, ERK5/BMK1. *Nucleic Acids Res*. 26, 4771-7.
- Youn, H. D., et al., 1999. Apoptosis of T cells mediated by Ca²⁺-induced release of the transcription factor MEF2. *Science*. 286, 790-3.
- Yu, Y. T., et al., 1992. Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes Dev*. 6, 1783-98.
- Zhang, C. L., et al., 2002. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell*. 110, 479-88.
- Zhang, D., et al., 2012. Discovery of Novel DENN Proteins: Implications for the Evolution of Eukaryotic Intracellular Membrane Structures and Human Disease. *Front Genet*. 3, 283.
- Zhang, Z. C., Chook, Y. M., 2012. Structural and energetic basis of ALS-causing mutations in the atypical proline-tyrosine nuclear localization signal of the Fused in Sarcoma protein (FUS). *Proc Natl Acad Sci U S A*. 109, 12017-21.
- Zhao, M., et al., 1999. Regulation of the MEF2 family of transcription factors by p38. *Mol Cell Biol*. 19, 21-30.
- Zhao, Y., et al., 2005. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature*. 436, 214-20.
- Zhou, X., et al., 2007. Characterization and identification of microRNA core promoters in four model species. *PLoS Comput Biol*. 3, e37.

Chapter 2 -

Study of a panel of miRNAs commonly dysregulated in different in vitro, in vivo and ex vivo ALS models

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Keywords: microRNA (miRNA), Amyotrophic Lateral Sclerosis (ALS), ELAVL4/HuD, PBMCs

2.1 Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that specifically affects upper and lower motor neurons leading to progressive paralysis and death. Currently, no effective treatments is available. Thus, the identification of the signaling pathways and cellular mediators involved in ALS remains a major challenge in the search for novel therapeutics.

Recent studies have shown that microRNA have a significant impact on normal central nervous system (CNS) development and on onset and progression of neurological disorders. Based on this evidences, in this study we tested the hypothesis that misregulation of miRNA expression could play a role in the pathogenesis of ALS. Hence, we exploited human neuroblastoma cell lines expressing SOD1(G93A) mutation as tools to investigate the role of miRNAs in familiar ALS. To this end, we initially checked the key molecules involved in miRNAs biogenesis and processing in these cells and we found a different protein expression pattern. Subsequently, we performed a genome-wide scale miRNA expression, using whole-genome small RNA deep-sequencing followed by quantitative real time validation. This strategy allowed us to find a small group of altered miRNAs, which were predicted to play a role in the motroneurons physiology and pathology. We measured this group of deregulated miRNAs by real time PCR in transgenic mice expressing the pathogenic protein SOD1(G93A) and furthermore in lymphocytes obtained from a group of sporadic ALS patients. We found that mir129-5p was up-regulated in cells, mice and in patients and we validated HuD as mir129-5p target. Previously, it has been reported that ELAVL4/HuD plays a role in neuronal plasticity, in recovery from axonal injury and multiple neurological diseases. Furthermore, we generated stable cell line over-expressing mir129-5p and we found a reduction in neurite

outgrowth and in the expression of differentiation markers with respect to control cells. Taken together these data strongly suggest that microRNAs play a role in ALS pathogenesis and in particular, that mir129-5p can affect neuronal plasticity by modulating ELAVL4/HuD level.

2.2 Introduction

ALS is a progressive, fatal neurodegenerative disorder characterized by loss of motorneurons from the spinal cord and motor cortex that leads to death within 2-3 years of onset (Cleveland and Rothstein, 2001). It is one of the most common motor neuron diseases occurring 1.7 ~ 2.3 out of 100,000 person per year worldwide (Beghi et al., 2006). The symptoms of ALS usually starts after 50 years of age, but it can also occurs in younger people. Usually, the disease onset occurs with the degeneration of specific subsets of motor neurons. Then, ALS progressively spreads to neighboring motor neurons and leads to atrophy of associated muscle tissues (Pratt et al., 2012). The genetic and environmental causes of ALS are still under investigation, but about the 90% of ALS cases are sporadic or caused by unknown genetic factors. So far about only 10% of cases can be traced to genetic factors (Al-Chalabi et al., 2012). The most well known genetic cause of ALS are mutations in Cu/Zn Super Oxide Dismutase 1 (SOD1) gene (Rosen et al., 1993). Only recently, with advanced genomic screening tools, several other genes associated to ALS have been identified including TAR DNA-binding protein (TDP-43), FUS, ALS 2 (ALS2), neurofilament heavy peptide (NEFH); (Al-Chalabi et al., 2012) and C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011). While C9ORF72 has been identified as the most prevalent mutation among ALS patients, with 40% of fALS patients carrying the mutation (Majounie et al., 2012), the abundance and variety of identified SOD1 mutations have made this a widespread experimental paradigm (Renton et al., 2011). Although the pathological characteristics of ALS are well defined, the cellular and molecular mechanisms linked to mutation of these genes and responsible of disease onset, are still under investigation.

MicroRNAs (miRNAs) are endogenous non-coding single-strand RNA molecules that play important roles in eukaryotic gene expression through posttranscriptional regulation (Bartel, 2009). They mainly bind to the 3'-untranslated region (3'-UTR) of messenger RNAs (mRNAs) and lead the target to gene silencing by mRNA cleavage, translational repression and deadenylation (Huntzinger and Izaurralde, 2011). MiRNAs are involved in a variety of physiological phenomena and diseases (Kim et al., 2009). In diseases affecting the central nervous system the loss of Dicer, a key regulator of miRNA biogenesis, induces neurodegeneration suggesting that miRNAs may play important roles in neurological disorders (Cuellar et al., 2008; Schaefer et al., 2007). Indeed, while the role of individual miRNAs in neurological disorders is not yet fully understood, there are growing evidences that miRNAs play a critical role in neurological disorders, such as miR-206/miR-153 in Alzheimer's disease (Lee et al., 2012; Liang et al., 2012), miR-34b/miR-9/miR-9* in Huntington's disease (Gaughwin et al., 2011; Packer et al., 2008), miR-128a/miR-24/let-7b in mood disorder (Zhou et al., 2009), miR-189 in Tourette's syndrome (Abelson et al., 2005), miR-9 in SMA (Haramati et al., 2010), miR-206/miR-338-3p/miR-451 in ALS (Butovsky et al., 2012; De Felice et al., 2012; Williams et al., 2009), miR-21/miR-431/miR-138 for axonal regeneration for sensory neurons (Liu et al., 2013; Strickland et al., 2011; Wu and Murashov, 2013), and miR-133b/miR-21 for spinal cord injury (Hu et al., 2013; Yu et al., 2011). However, pathological contribution of individual miRNAs to each of these diseases is still under investigation. Especially, our knowledge about the role of miRNAs in motor neuron diseases is very limited. This could be due to the complexity of the nervous system and the technical difficulties of studying neurological disorders. MicroRNAs have also demonstrated their potential as non-invasive biomarkers from blood and serum for a wide variety of human pathologies as

well as neurodegenerative diseases (Keller et al., 2011). Concerning ALS, miRNAs expression in white blood cells from sporadic ALS patients appears to exhibit distinct expression patterns during disease progress. miRNA profiling data from 14 patients and 14 controls showed that expression of miR-338-3p is increased and expression of seven other miRNAs is decreased in leukocyte from ALS patients (De Felice et al., 2012). Moreover, another recent publication reports that miR-206 was found increased in the circulation of symptomatic animals and in a group of 12 definite ALS patients and it suggests that may be an interesting potential candidate as a biomarker. To further characterize the roles of miRNAs in ALS pathogenesis and their potential use as biomarkers and, hopefully, also targets for new therapy in ALS, an accurate profiling of miRNAs expression in different experimental ALS models is necessary. Here, we present a characterization of miRNAs expression in a cellular SOD1(G93A) ALS-linked model. First, we have analyzed by Western Blot the expression of crucial factors involved in miRNA biogenesis and processing and we have observed an altered expression pattern between mutated cells and control cells. Afterwards, we used the RNA high throughput sequencing to obtain the miRNA profile of SOD1(G93A) cells and we identified a small subset of deregulated miRNAs that we have subsequently analyzed in SOD1(G93A) mice. In order to identify a common signature between familial and sporadic ALS, we assessed the expression level of mir129-5p and mir200c, which we previously found upregulated in SOD1(G93A) cells as well as in mice, in PBMCs obtained from sporadic ALS patients. We observed that mir129-5p was upregulated in cells, mice and PBMCs and we validated ELAVL4/HuD as novel mir129-5p target. Furthermore, we generated stable cell line overexpressing mir129-5p and we found a reduction in neurite outgrowth and in the expression of differentiation markers in compare to control cells. Taken

together these data strongly suggest that microRNAs play a role in ALS pathogenesis and in particular, that mir129-5p can affect neuronal plasticity by modulating ELAVL4/HuD levels.

2.3 Materials and Methods

RNA high throughput sequencing

Human neuroblastoma SHSY-5Y cells untransfected or stably transfected with cDNA coding for wild type SOD1 or the mutant SOD1(G93A) were sequenced by whole-genome small RNA deep-sequencing (sRNA-seq) using Solexa system. For the statistical analysis (performed by Dr. R. Calogero, Bioinformatics and Genomics Unit, University of Turin) were considered only those reads that will be greater than 10 nucleotides.

Cell cultures, transfections and drug

Human neuroblastoma SHSY5Y cells untransfected or stably transfected with cDNA coding for wild type SOD1 or the mutant SOD1(G93A) were maintained in Dulbecco's modified Eagle's Medium supplemented with antibiotics (100 U/mL streptomycin and 100 µg/ml penicillin), 2,5 mM L-Glutamine and 10% Fetal Bovine Serum at 37 °C with 5% CO₂. SOD-1 stably transfected cells were also maintained in the presence of 400 µg/mL of Geneticin (G418 sulphate, prepared as 40 mg/ml stock solution in water). Cells were fed every 2-3 days and passed once a week. All reagents were supplied by Euroclone.

For the luciferase assay experiments, 1,5 X 10⁵ SHSY5Y cells were seeded in a 24 multiwell, and the next day plasmids (a total of 1,2 µg) were co-transfected using Polyethylenimine (PEI, Sigma, 100 mM in H₂O pH 7.00) according to the manufacturer's instruction.

For differentiation treatment, cells were incubated with Retinoic Acid 10uM for 6 days before collecting.

PBMCs isolation

PBMCs were isolated from whole blood by Ficoll-Histopaque (GE Healthcare) density gradient centrifugation. Briefly, blood samples were diluted with the same amount of saline solution, layered on Ficoll-Histopaque and centrifuged (490xg, 30 min, room temperature). PBMCs were collected from the interface between plasma and Ficoll-Histopaque, washed with saline solution, aliquoted and stored at -80°C.

RNA extraction reverse-transcription and Quantitative Real-Time PCR assay (qPCRs)

SYSH5Y cells were seeded on 10 cm plates, and 24 hours after plating RNAs were extracted using TRIzol Reagent (Invitrogen) and subsequently purified using silica membrane spin columns from RNeasy Mini Kit (Quiagen). RNA quantity and purity were assessed using a NanoDrop instrument (Thermo Fisher Scientific Inc.). 2 µg of total RNA were reverse-transcribed using the random hexamers-based High Capacity cDNA Reverse-Transcription Kit (Applied Biosystem), according to manufacturer's instructions.

In order to validate the microarray data regarding HOXC10, CACNG2 and ELAVL4 gene levels downregulation, qPCRs were performed in a final volume of 20 µl with SYBR Green qPCR master mix (Applied biosystem), 1 µl cDNA diluted (1:50), and 0,5 µM of each primer. The primers used for CACNG2 genes were: forward: 5'CTCTCTACTCCCACCCCTTG3'; reverse: 5'GCTCTGCTCCGTCTTGATT3'; for HOXC10 were: forward: 5'ACTCGAGAAGATGCCGGATAATCGG3'; reverse: 5'ATCTAGTCAGCTGTCAAGAGGAAGC3'.

Normalization of cDNA loading was obtained running all samples in parallel using human GAPDH as housekeeping gene. The primers for GAPDH were: forward: 5'ACGGATTGGTCGTATTGGG3'; reverse: 5'TGATTTGGAGGGATCTCGC3'. The amplification protocol was as follow: an initial denaturation and activation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Normalization of the target amplification over the normalize was performed using Excel software.

miRNA extraction, reverse transcription and qPCR for SHSY5Y cells and PMBCs

The pellet of PMBCs was lysed by adding 1 ml of Trizol. In order to obtain a microRNA-enriched fraction the aqueous phase was processed using the Absolutely RNA miRNA kit (Agilent Technologies) according to the manufacturer's protocol.

The microRNA from SHSY5Y cells were extracted using the Absolutely RNA miRNA kit (Agilent Technologies) according with the protocol.

2 µg of miRNA were used for retro-transcribing reaction that was performed using an homemade miRNA detection kit. Essentially in the retrotranscription reaction a poly-A tail is added to the mature microRNA template by E. coli Poly(A) polymerase (New England Biolabs); cDNA is synthesized by the Affinity-script polymerase (New England Biolabs), using a poly-T primer with a 5' universal tags and a 3' a degenerate anchor. The cDNA template is then amplified using a micro-RNA specific forward primer and a universal reverse primer (that is complementary to the universal tag). SYBR Green (MESA GREEN qPCR MasterMix Plus) is used for detection.

List of specific forward primers:

Hsa-mir30a	Hsa-mir124
ACATCCTCGACTGGAAGAA	CACGCGGTGAATGCCAAA
Hsa-mir30b	Hsa-mir125a-3p
TGTAAACATCCTACACTCAGCTAA	GTGAGGGTTCTTGGGAGCCAAA
Hsa-mir30c	Hsa-mir125-5p
TGTAAACATCCTACACTCTCAGCAA	CTGAGACCCTTAACCTGTGAAA
Hsa-mir30d	Hsa-mir125b
CATCCCCGACTGGAAGAA	CCCTGAGACCCTAACCTGTGAAA
Hsa-mir30e	Hsa-mir-132
AACATCCTGACTGGAAGAA	CAGTCTACAGCCATGGTCGAAA
Hsa-mir29a	Hsa-mir17
CACCATCTGAAATCGGTTAA	GTGCTTACAGTGCAGGTAGA
Hsa-MIR29b	Hsa-miR7
TAGCACCATTGAAATCAGTGTAA	TGGAAGACTAGTGATTTGTTGTAAA
Hsa-mir1	Hsa-mir200C
TGGAATGTAAGAAGTATGTATAA	TAATACTGCCGGGTAATGATGGAAAA
Hsa-mir206	Hsa-mir885-3p
TGGAATCTAAGGAAGTGTGTGGAA	AGGCAGCAGGGGTGTAGTGGATAAA
Hsa-mir15a	Hsa-mir455-5p
GCAGCACATAATGGTTGTGAAA	TATGTCCTTGGACTACATCGAAA
Hsa-Mir15B	Hsa-Mir9
GCAGCACATCATGGTTACAAA	TCTTGGTTATCTAGCTGTATGAAA
Hsa-mir16	Hsa-mir455-3p
CAGCACGTAATATTGGCGAA	GCAGTCCATGGCATATTCAAA
Hsa-mir195	Hsa-mir1179
TAGCAGCACAGAAATATTGGCAA	AAGCATTCTTCATTGGTTGGAAA
Hsa-mir203	Hsa-mir1287
GTGAAATGTTAGGACCACTAGAAA	TGGATCAGTGGTTCGAGTCAAA
Hsa-Mir9	Hsa-mir1179
TCTTGGTTATCTAGCTGTATGAAA	AAGCATTCTTCATTGGTTGGAAA
Hsa-mir455-3p	Hsa-mir1287
GCAGTCCATGGGCATATTCAAA	TGGATCAGTGGTTCGAGTCAAA
Snord25	Snord25
GATGAGGACCTTTCACAGACCTG	GATGAGGACCTTTCACAGACCTG

SHSY5Y data were normalized on SnoRD25, mice miRNAs were normalized on RNU1A1 while PMBCs miRNA expression were normalized on mir-16 and Snord25 expression level.

Extraction, Reverse transcription and qPCR for RNA extracted from spinal cord of ALS mice.

The RNA isolation was carried out using TRIZOL reagent retrotranscribed according to the protocol. 5 ng of template RNA samples were amplified using the miRCURY LNA TM Universal RT microRNA PCR (Exiqon) according to the manufacturer's protocol. Quantitative PCR was performed on a customized Pick & Mix PCR panels which consist of 96-well PCR plates containing a selection of dried down microRNAs LNA TM PCR primer sets.

Plasmids and cloning

The pre-miR 129 expressing plasmid in pMir and the HOXC10 3'UTR Reporter Clone in pMir Target were purchased from Origene. The CACNG2 and ELAVL4/HuD UTR regions were amplified using the Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) according to manufacturer's instructions. The human genomic DNA extracted from HeLa cells was used as the template in the PCR reactions. The sequences of the oligos used in this PCR reaction were: CACNG2 UTR forward: 5'TCCACTCCAACACAGCCAAC3'; Reverse: 5'CTTACGCGTTGTTTCTTCCTCGTTA3'. ELAVL4/HuD UTR Forward: 5'CCAACAAAGCCCACAAGTCCTGA3'; Reverse: 5'ATTACGCGTATGTCATCAGGTATCCCCCT3'. The resulting PCR products were purified and cloned into the pGEM T Easy Vector system (Promega). The insert was verified by nucleotide sequencing.

Bioinformatic miRNA target prediction

The target mRNA that have the potential binding sites for individual miRNAs were identified by searching them on public databases endowed with prediction algorithms, such as TargetScan, PicTar, miRBase, Miranda, Diana Lab.

Protein extracts and immunoblotting

Cells on 10 cm plate were washed once in PBS 1X (Euroclone) and then lysed in 100 µl of cold Lysis Buffer (Tris HCl 50 mM pH 7,5, NaCl 150 mM, 1% NP40, 5 mM EGTA) with protease inhibitors (Roche) and phosphatase inhibitor (Sigma-Aldrich). The samples were put on ice for 20 min and then centrifuged at 15000 rcf for 15 min at 4°C, and the

supernatant were collected. An aliquot of the cell lysate was used for protein analysis with the Comassie protein assay reagent for protein quantification.

Protein were separated in 8% and 10% SDS-polyacrylamide gels (classic Laemli conditions) and transferred to nitrocellulose membranes (Whatman GmbH), in normal Transfer Buffer (25 mM Tris, 192 mM Glicine, 20% Methanol) at 100 Volts for 2h or in High Molecola Weight protein (25 mM Tris, 192 mM Glicine, 10% Methanol and 0,1% SDS) at 15 Volts overnight. Membrane were blocked using 5% non fat dried milk in PBST (0,1% (v/v) Tween 20 in 1X PBS) for 1h at room temperature or overnight at 4 °C. After washing 3 times with PBST, membranes were incubated with peroxidase-conjugated secondary antibody anti-mouse IgG (Cell signaling, 1:3000 dilution) or anti-rabbit IgG (Cell signaling 1:8000 dilution), in PBST with 5% non fat dried milk for 1h at room temperature.

After washing as above, the chemio-luminescent signals developed by ECL reagents (Millipore) were detected using Li-cor technology Biosciences.

Quantification of signals were performed using Li-cor technology software, each band value were normalized on the corresponding housekeeping band intensity, thus obtaining relative intensity values.

Antibodies

The antibodies used for immunoblotting were: Rabbit polyclonal anti Hoxc10 (AVIVA ARP39274_P050 1:1000 dilution); rabbit polyclonal anti CACNG2 (AVIVA ARP35189_050 1:1000 dilution); mouse monoclonal anti HuD (SC48421, 1:2000 dilution); goat polyclonal anti LDH (Abcam 1222 1:1000 dilution); goat polyclonal anti Drosha (sc31159, 1:500 dilution); mouse monoclonal anti Dicer (sc136980 1:500

dilution); rabbit polyclonal anti TAF15 (bethyl A300-308 1:1000 dilution); rabbit polyclonal anti EWS (bethyl A300-418 1:1000 dilution); mouse monoclonal TDP43 (sc100871 1:1000 dilution); rabbit polyclonal anti FUS (home.made 1:5000 dilution); rabbit polyclonal anti Exportin 5 (sc-66885 1:5000 dilution). All the antibodies were diluted in PBS-Tween 0,01%- MILK 5%.

Luciferase reporter assays

1,5 X 10⁵ human neuroblastoma SHSY5Y cells, stably over-expressing the SOD1-WT or the SOD1(G93A) variant, were seeded in 24 multiwells as described above. 24h hours after seeding, cells were co-transfected with a total of 1,2 µg of plasmid DNA composed of a constant amount of Renilla expression vector pRL-TK (50ng/well, Promega) and a variably amount of pMiR vector containing the pre-miRNA and a variably amount of 3' UTR Reporter Clone in pMir Target containing the Firefly luciferase which expression is driven by SV40 Promoter. The two vectors were transfected in different proportion: 1:3, 1:1, 3:1. The experiment were carried out in duplicate. Luciferase expression was maintained for 24 hours, and then cells were lysed in 100 µg/well of the Passive Lysis Buffer 1X according to manufacturer's instructions. To obtain a complete lysis, cells were subjected to a freeze-thaw cycle. 10 µl of lysates were used to the luciferase assays, which was carried out using the Dual-Luciferase Reporte Assay System (Promega) and a Berthold luminometer (Berthold Inc.) The relative luminescence units (RLUs) were obtained normalizing the Firefly luciferase reading to the corresponding Renilla luciferase readings.

2.4 Results

Proteins involved in miRNAs biogenesis and processing are differentially expressed in SHSY5Y SOD1(G93A) cells.

It has been previously reported that in different neurodegenerative disorders there is an impairment of proteins involved in miRNAs biogenesis and processing (Bicchi et al., 2013). Moreover, two ALS associated genes FUS and TDP43 are directly involved in miRNAs biogenesis pathway (Gregory et al., 2004). In particular way, TDP-43 is a component of the Dicer and Drosha complexes and more recently, it has been shown that FUS promotes biogenesis of specific miRNAs via the recruitment of Drosha to primary miRNA transcripts. In order to unravel the impact of miRNAs in ALS pathogenesis we exploited human neuroblastoma cell lines expressing SOD1(G93A) mutation and SOD1-WT as ALS familiar model. First, we analyzed by Western Blot analysis the protein levels of some of the key players involved in miRNA biogenesis and processing (Figure 1A and 1B). In particular, we analyzed Drosha and its cofactor DCRG8, that process pri-miRNA in pre-miRNA and we found a slight decrease of about 0,5-1,5 fold change for both proteins in mutant cells. EWS and TAF15, the two proteins that together with FUS compose the FET family (known Drosha interactors), were found increased of about 1-2 fold change in SOD1(G93A) cells in comparison with wild type cells, while FUS and TDP43 do not show any change. Intriguingly, we found different proteins level also concerning Dicer, the RNaseIII enzyme that leads pre-miR to mature microRNA, that resulted 2-3 fold change increased. We analyzed also the protein level of Exportin 5, but we did not find any changes. These results shows that in our model system there is an impairment of the biogenesis and processing complex that realistically has an impact on

miRNA levels, but further analysis will be necessary to understand the mechanisms underlying this alteration.

Figure 1

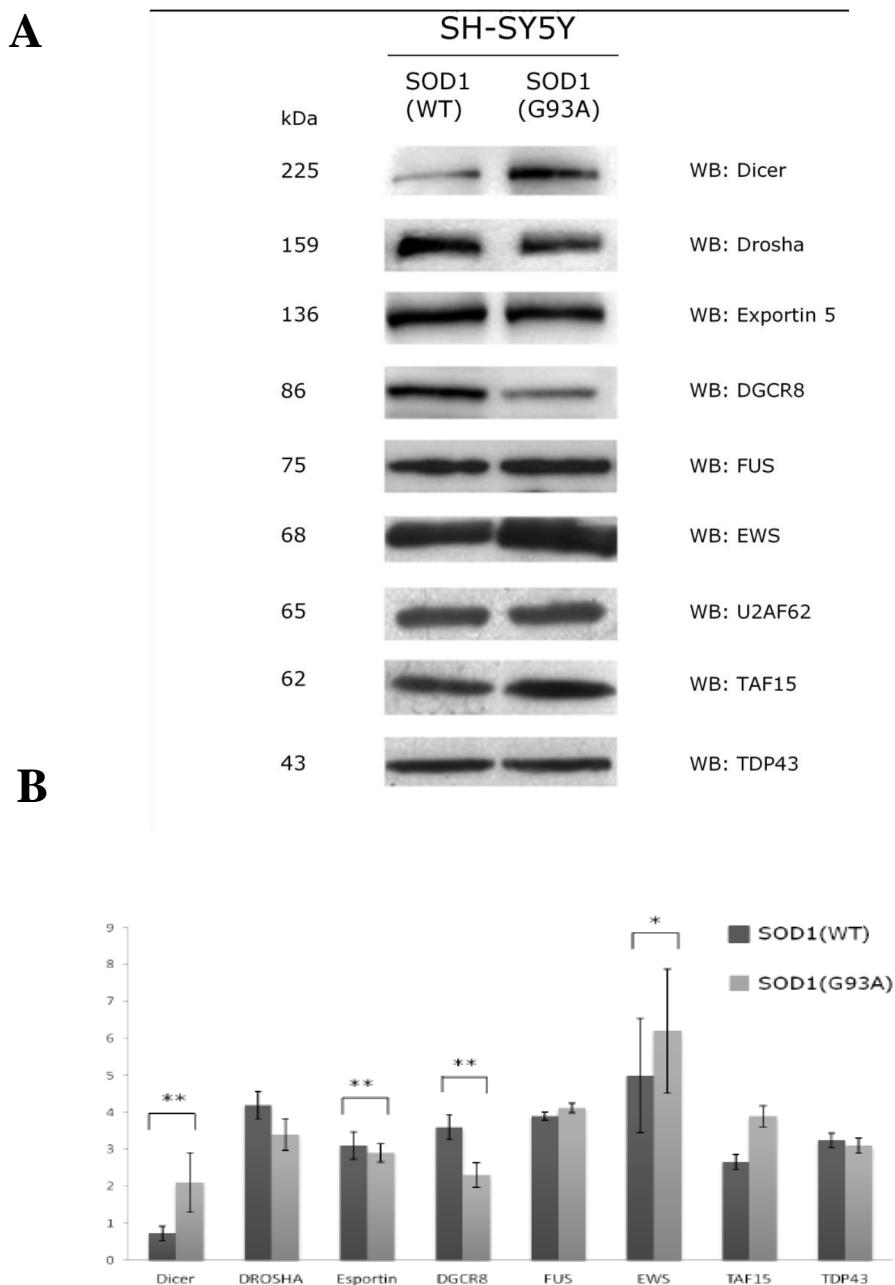


Figure 1: Analysis of proteins involved in microRNA biogenesis by Western Blot. (A) In the first and second lane are shown protein levels of SHSY5Y SOD1-WT and SOD1(G93A) respectively. Proteins were detected using Li-cor Technology. (B) Semi-quantification of proteins detected. Data were normalized to GAPDH amount for protein with low molecular weight and vinculin for protein with high molecular weight.

The expression of a subset of miRNAs is modified in an *in vitro* ALS model.

The different protein pattern of miRNA regulators suggested a misregulation in miRNAs expression. Different approaches can be used to assess microRNAs. As a first screening, we used a computational identification inferred from the expression changes in their putative mRNA targets. Previously, in the laboratory of Prof. Barabino, it was performed a whole-genome splicing sensitive microarray analysis of human neuroblastoma cell line (SHSY5Y) stably expressing wild-type SOD1 (SOD1-WT) versus cells expressing a pathogenic mutation (SOD1-G93A) (Lenzen et al., 2011). This analysis allowed the definition of both transcription patterns (gene-level analysis) and the alternative pre-mRNA maturation events (exon-level analysis). The results obtained revealed that profound changes exist in the expression of genes involved in relevant pathways for nervous system development and function such as axon guidance and growth, synaptic vesicles formation and recycling, cytoskeleton structure and movement. Thus, we used the T-REX (Target Reverse Expression) algorithm to identifies regulated miRNAs via the modulation of their predicted targets (Volinia et al., 2010). Essentially, this algorithm is based on the assumption that when a miRNA is active in cells, its target is repressed and vice versa, if the targets are over-expressed the controlling miRNA is down-regulated. This computational approach identified 54 (Table 1) putative differentially expressed miRNAs in SH-SY5Y SOD1(G93A) cells. As a next step, we performed a Next Generation high throughput Sequencing (miRNA-seq) on the same cell lines and we found 9 differentially expressed miRNA (Table 2) between mutant SHSY5Y with respect to control cells. To validate both the T-REX and the NGS output, we carried out quantitative real time PCRs (qPCR) and we confirmed the differential expression of a group of miRNAs found altered by both NGS and T-REX list in line with the qPCR

validation (Figure 2). In particular, we observed the up-regulation of mir129-5p (six fold change), mir-200c (three fold change) and mir-7 (nearly two fold change); and the down-regulation of mir-124 and mir-455 (about 0.2 fold change for both).

Table 1

MI-RNA	Down-regulated	Up-regulated
miR-30a-5p	0,00079	0,09218
miR-30b	0,00079	0,09218
miR-30c	0,00079	0,09218
miR-30d	0,00079	0,09218
miR-30e-5p	0,00079	0,09218
let-7a	0,00264	0,72438
let-7b	0,00264	0,72438
let-7c	0,00264	0,72438
let-7e	0,00264	0,72438
let-7f	0,00264	0,72438
let-7g	0,00264	0,72438
let-7i	0,00264	0,68389
miR-146a	0,00264	0,25051
miR-146b	0,00264	0,25051
miR-29a	0,00264	0,10903
miR-29b	0,00264	0,10903
miR-29c	0,00264	0,10903
miR-98	0,00264	0,72438
miR-1	0,00455	0,14317

(S. Volinia, University of Ferrara)

Table 1: Computational identification of miRNAs. Splicing-sensitive Microarray Analysis of Models of Mitochondrial Stress (SHSY5Y SOD1-G93A cells) showed profound changes in the expression of genes involved in relevant pathways for neuron survival at gene-level and in pre-mRNA splicing. T-REX algorithm identified regulated miRNAs via the modulation of their predicted targets. 54 putative miRNAs differentially expressed in SHSY5Y SOD1(G93A) cells were identified.

Table 2

ENSEMBL ID	miR16 precursor	Log2 FC
ENSG00000199077	hsa-mir-129-2	UP mt
ENSG00000207598	hsa-mir-124-3	DW mt
ENSG00000207705	hsa-mir-129-1	UP mt
ENSG00000207703	hsa-mir-7-2	UP mt
ENSG00000207713	hsa-mir-200c	UP mt
ENSG00000221419	hsa-mir-1287	UP mt
ENSG00000221630	hsa-mir-1179	UP mt
ENSG00000216135	hsa-mir-885	DW mt
ENSG00000207726	hsa-mir-455	DW mt

(R. Calogero, University of Turin)

Table 2: Next generation High throughput sequencing ouput. The RNA sequencing showed 9 differentially expressed miRNA between SHSY5Y expressing mutant SOD1 protein with respect to controls.

Figure 2

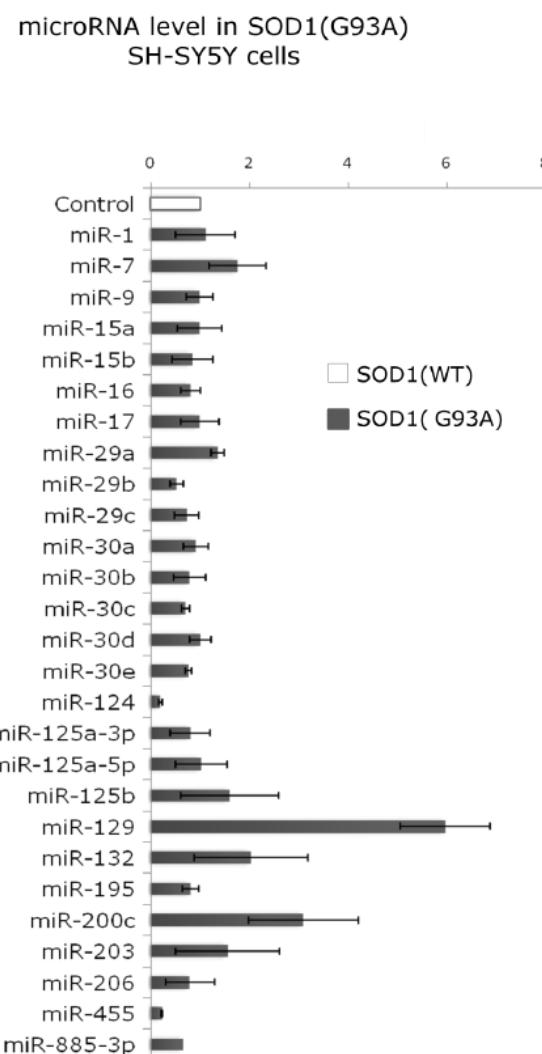


Figure 2: Validation by RT-qPCR of the top ranking up- or down-regulated miRNAs identified by T-REX and those identified by RNAseq. Experimental validation of altered miRNAs identified by T-REX and by RNA-seq by qPCR (n=3 independent experiments) in SHSY5Y cells stably expressing SOD1 WT (in white) and in SHSY-5Y stably expressing SOD1(G93A) (in dark grey). Data were normalized to snoRD25.

Characterization of miRNAs expression in G93A mice

In order to investigate if the expression of the misregulated miRNAs, identified in a cellular model, could be affected also in a model more related to the pathology, we analyzed their levels in transgenic mice over-expressing the SOD1-G93A mutation. 18 of the differentially expressed miRNAs were analyzed on RNA extracted from spinal cord of 8 transgenic mice carrying the SOD1-G93A. In particular, six mice were at the early stage (130-135 days) and two were at symptomatic stage (140-170 days) of the disease. As a control, we used 8 normal littermates of the corresponding age group. Our results at the early stage of disease showed increased levels of mir129-5p and mir-200c, although showing a lower fold change induction and variability with respect to mir-7 (Figure 3A). Instead, the down regulation of mir-124 previously found in SHSY5Y cells was not comparable in mice. Moreover, we found an up-regulation of mir-455 which was found to be downregulated in neuroblastoma cells. Surprisingly, at the symptomatic stage (Figure 3B) we found a slight global down-regulation of each miRNA making most of them again similar to the control mice, with the exception of mir-200c that is drastically reduced. These results may suggest that miRNA expression may vary in a stage specific manner. In particular, way mir129-5p showed a strong increase at the early stage and its levels decreased with the progression of disease suggesting a potential role in the disease onset.

Figure 3

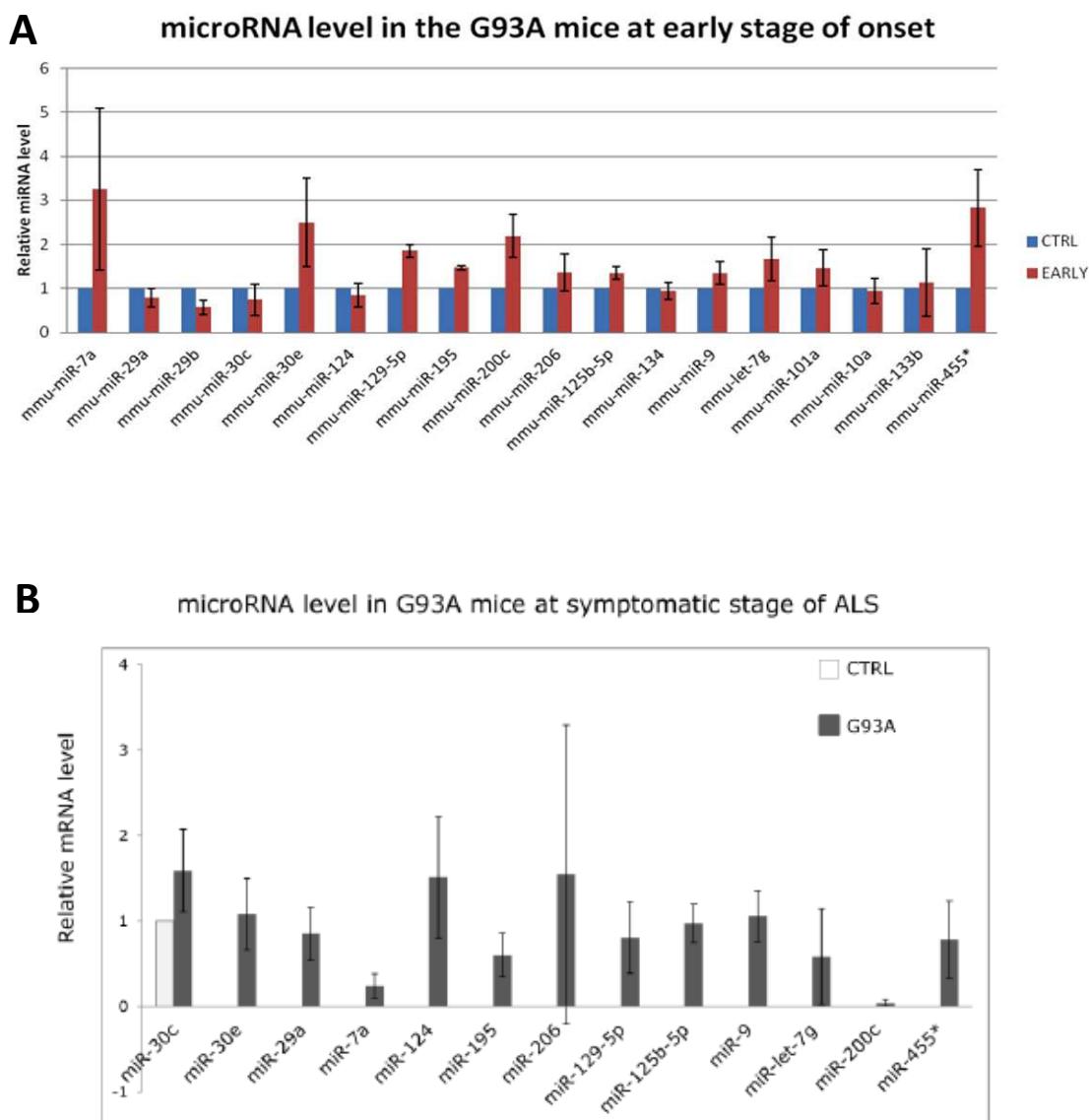


Figure 3: Analysis of misregulated microRNA in ALS mice. Analysis of misregulated microRNA in mice (n=6 biological replicates) carrying the SOD1(G93A) at the early stage (130-135 days) of disease (A); and at the symptomatic stage (140-170 days), (n=2 biological replicates) (B). Data were normalized to RNU1A1.

Mir-129-5p and mir-200c are upregulated in PBMC of sporadic ALS patients

Our result suggested a potential role of mir129-5p and mir200c in ALS pathogenesis. The increasing relevance of characterization of miRNAs profiling in biofluids in different neurodegenerative pathologies has prompted the study of these misregulated miRNAs in peripheral blood mononuclear cells (PBMCs) of sporadic ALS patients by qPCR. In particular, we analyzed samples of 28 patients and 22 controls taken from healthy volunteers (Figure 4). Interestingly, we found a significant up-regulation (2-fold change) of mir129-5p and mir200c in patients' samples. While the expression of mir-200c showed a higher standard deviation, mir129-5p had a good reproducibility among patients. Based on these results we decided to further characterize mir129-5p investigating its putative targets and the role that they may potentially play in disease onset and progression.

Figure 4

A

	ALS (n=28)	CTRL (n=22)
Age	63,6 ± 12,6	62,9 ± 12,0
Gender (M/F)	15/13	11/11
Form (Spinal/Bulbar)	20/8	n/a
ALSFRS-R	23,3 ± 7,2	n/a
Disease duration (months)	32,5 ± 30,2	n/a

B

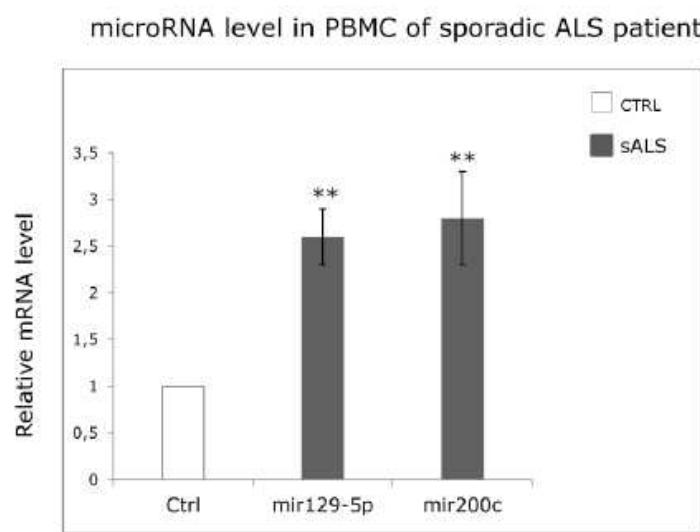


Figure 4: Analysis of the expression of misregulated miRNA in patients. (A): Clinical characteristics of the recruited samples. (B): Analysis of the expression of the top up-regulated miRNAs on RNA extracted from peripheral blood mononuclear cells (PBMCs) of sporadic ALS patients. Data were normalized to mir16 or Snord25 expression. (** p<0.001)

Mir-129-5p predicted targets

The human genome encodes over 1000 miRNAs that collectively target the majority of mRNAs. Base pairing of the so-called miRNA ‘seed’ region with mRNAs identifies many thousands of putative targets. The vast majority of predicted targets, often with quite disparate functions, make a challenge choosing which is worthy of experimental follow-up. To address this challenge, predictors may use thermodynamic, evolutionary, probabilistic or sequenced-based features. So far, the best strategy to pullout good candidates is to compare which targets have a high score for every one of these features.

In order to define the mechanism by which mir129-5p up-regulation can contribute in ALS pathogenesis we looked at its bioinformatic-predicted targets. Following the criteria mentioned above and wondering which targets could be significantly involved in ALS pathology, we selected four potential targets that are shown in the Table 3 with a short description. Starting from the output of the microarray profiling experiment, initially we decided to investigate the genes whose expression was found to be down-regulated in our system: HOXC10 and CACNG2 genes. HOXC10 belongs to the homeobox family of transcription factors. We selected this gene because it has been demonstrated that mutant mice (HoxC10^{-/-}) have a reduction in lumbar motor neurons (Hostikka et al., 2009). CACNG2 (Calcium channel, voltage-dependent, gamma subunit 2) also known as stargazin is involved in the transportation of AMPA receptors to the synaptic membrane, and the regulation of their receptor rate constant (Chen et al., 2000). We decided to investigate this putative target for the well-known implication of AMPA in ALS pathogenesis. Moreover, we decided to further analyze the ELAVL4/HuD transcript which was not present in the output list of the microarray for the well characterized role of HuD in neuronal cell identity, maturation and survival. As a first step, we validated the

microarray expression data for these genes by qPCR and checked for ELAVL4/HuD transcript levels. This validation showed a good correlation with the microarray data and confirmed the down-regulation of the three transcripts (Figure 5).

Homeobox protein Hox-C10	It has been demonstrated that mutant mice have a reduction in lumbar motor neurons
Calcium channel, voltage-dependent, gamma subunit 2 (CACNG2)	CACNG2 also known as stargazin is involved in the transportation of AMPA receptors to the synaptic membrane, and the regulation of their receptor rate constant
ELAVL4	The HuD/ELAVL4 protein is an RNA binding protein. It is expressed only in neurons and it binds to AU rich-element containing mRNAs. As a result of this interaction the half-life of the transcript is increased. HuD is important in neurons during brain development and plasticity.
VCP	The protein encoded by this gene is a member of a family that includes putative ATP-binding proteins involved in vesicle transport and fusion. VCP mutations may account for approximately 1%–2% of familial ALS.

Table 3: List of potential target of mir129-5p

Figure 5

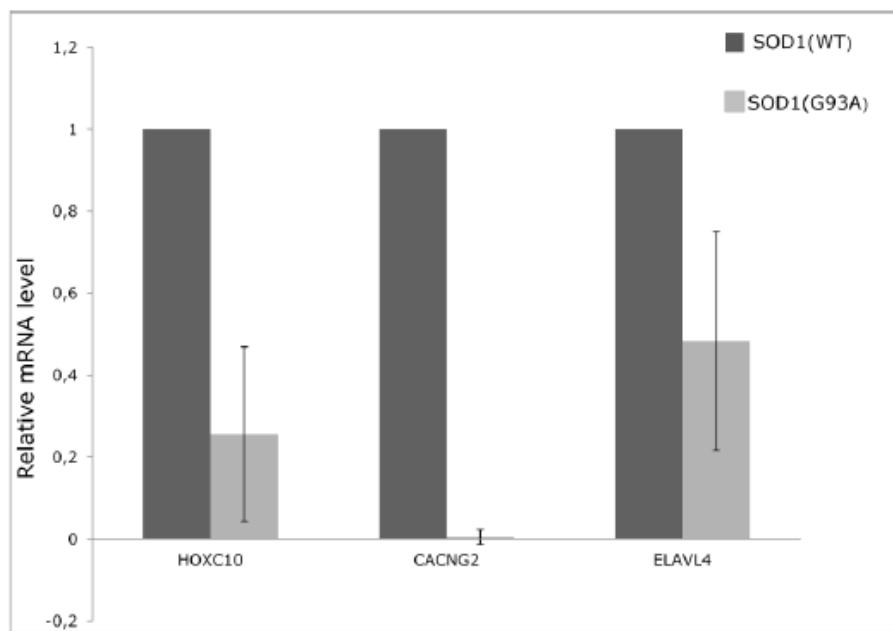


Figure 5: Analysis of the expression of miRNA targets. Results of RT-qPCR carried out on SHSY5Y cells over-expressing the SOD1 wild type or the mutant SOD1(G93A) in order to confirm the down-regulation of the putative mir129-5p targets.

ELAVL4/HuD is targets of mir-129-5p

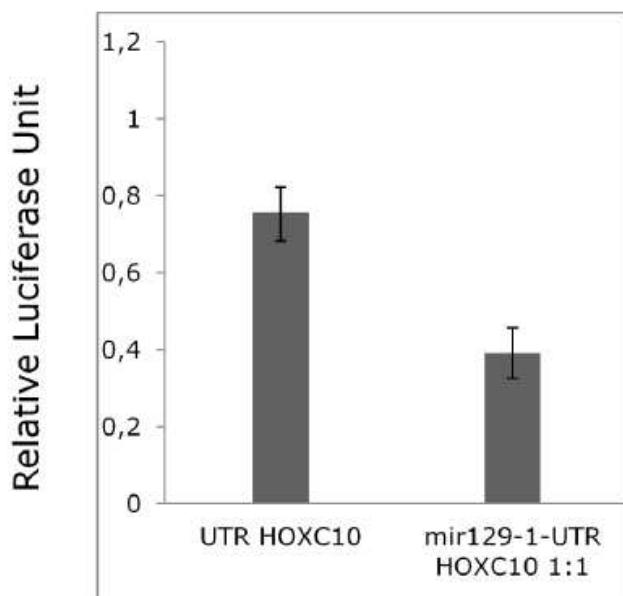
In order to validate the putative targets of mir129-5p by bionformatic analysis, we performed a luciferase assay. We used a plasmid that express the pre-miR129 sequence and a plasmid with the UTR of the gene cloned upstream of the luciferase gene (in which we cloned the 3'-UTR of the predicted target). After a transient co-transfection of both plasmids we tested the ability of mir129-5p to bind the 3'-UTR of the considered gene by luciferase assay. We first focused on HoxC10. The bionformatic analysis of this region identified one “conserved sites” for mir129-5p consisting in a “8mer seed match” that means an exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'. As shown in Figure 6A, the luciferase activity decrease in response to the presence of miR129-5p with a molar ratio of 1:1 between microRNA and UTR. Next, we cloned the 3'-UTR of CACNG2. The sequence of UTR has four conserved sites, one of them is “8mer” and three are “7mer-m8” that means an exact match to positions 2-8 of the mature miRNA (the seed + position 8). Finally, we cloned the 3'-UTR of ELAVL4/HuD that shows only one “7mer-m8” conserved seed sequence. As shown in Figure 6B e 6C, for both genes, the luciferase activity decreased in response to the presence of miR129-5p with a molar ratio of 1:1 between the microRNA and UTR of both sequences. These results show that there is a decrease of luminescence activity for every UTR of almost 40% for ELAVL4/HuD up to the 70%, for CACNG2, suggesting that the three genes examined were targets of mir129-5p.

Since, there are controversial opinions regarding the use of the luciferase assay to test the “miRNA-target interaction” because the transfection is only about the UTR sequence so it is not possible to measure the available of seed sequence and the thermodynamic

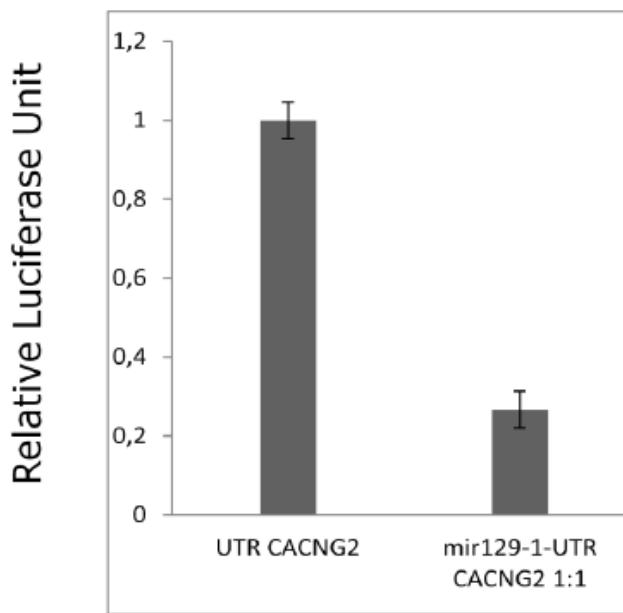
features. Thus, this method may be considered a way to force the interaction. To escape these controversies and be more confident about the targets, we generated a stable cell line that over-express pre-mir129-1 and we measured the endogenous protein level of the putative targets we selected. As shown in Figure 7 the Western Blot analysis revealed that only ELAVL4/HuD protein level is really affected by the mir129-5p over-expression (almost 60% of decrease), while stargazin presented only a slight decrease and HOXC10 an up-regulation. These results showed that even if mir129-5p is able to bind the 3'-UTR of the three genes, only ELAVL4/HuD endogenous protein is strongly affected by the microRNA over-expression.

Figure 6

A



B



C

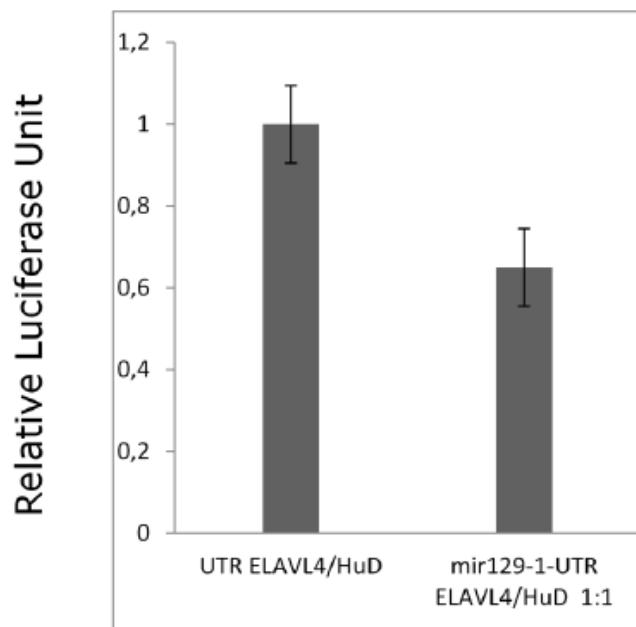


Figure 6: Validation of putative targets of mir 129-5p by luciferase assay. The first histogram shows the luminescence due to the luciferase gene expressed from the the UTR plasmid. The second shows the reduced level of luminescence in presence of mir129-5p precursor. The amount of microRNA and UTR is 1:1. **(A)** Shows the reduction of luminescence due to the binding of mir129-5p to HOXC10 UTR in SHSY5Y cells. **(B)** Shows the reduction of luminescence due to the binding of mir129-5p to CACNG2 UTR in HEK cells. **(C)** Shows the reduction of luminescence due to the binding of mir129-5p to ELAVL4/HUD UTR in HEK cells.

Figure 7

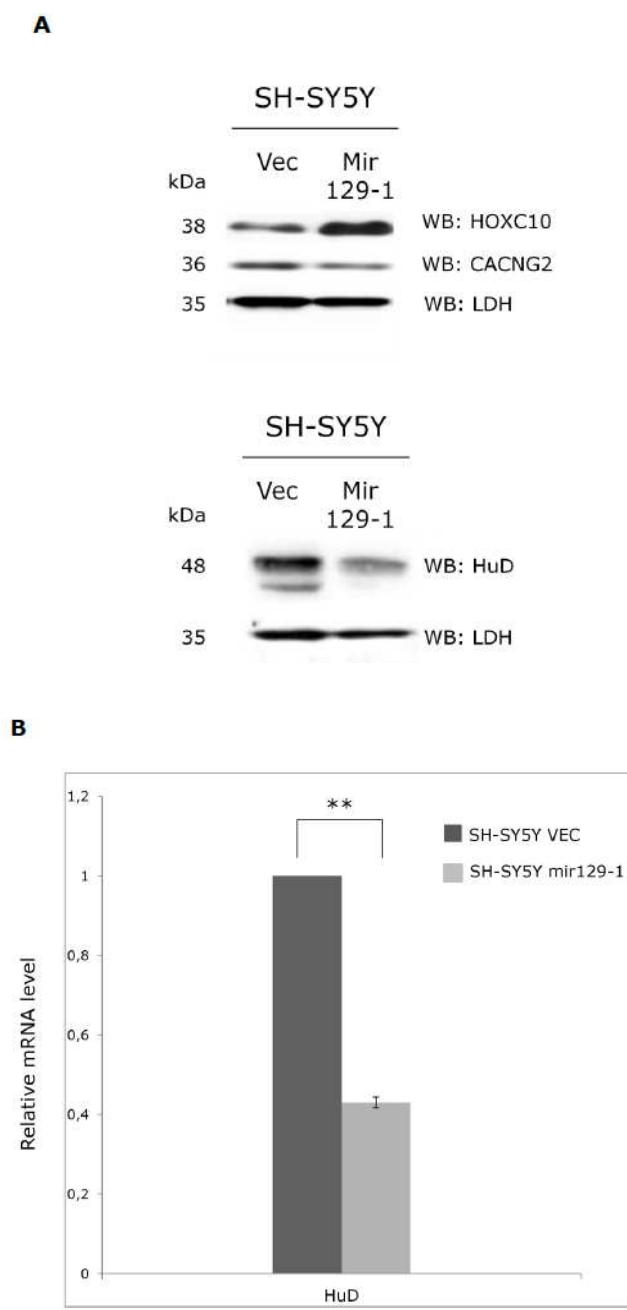


Figure 7: Validation of the putative targets of mir129-5p by western blot. (A) In the first and second lane are shown proteins from total lysates of cells transfected with the empty vector (Vec) and with pre-mir129-5p (mir129-5p). The protein analysed were the endogenous HOXC10, CACNG2 and HuD. **(B)** Relative quantification of proteins detected. Data were normalized on LDH levels.

The overexpression of pre-mir129-1 inhibits neurite outgrowth and differentiation

Our data strongly suggested that ELAVL4/HuD is target of mir129-5p. Since this protein is strongly expressed within the central nervous system and regulates posttranscriptionally several mRNAs that codify for proteins playing key roles in neurons, it was hypothesized that ELAVL4/HuD could acts as a “master regulator” of various neuronal processes (Deschênes-Furry et al., 2006; Perrone-Bizzozero and Bird, 2013). Moreover, many studies evidenced its importance in neuronal function and survival, as well as plasticity during learning and memory and following neuronal injury. Additionally, there is accumulating support implicating misregulation and mutation of ELAVL4/HuD in neuronal pathologies and neuroendocrine cancers. Moreover a previous publication indicates that ELAVL4/HuD down-regulation is able to inhibit the differentiation of neurites (Abdelmohsen et al., 2010). Based on these reports and in order to characterize the phenotypical effect of mir129-5p over-expression, we checked and measured the neurites outgrowth of SHSY5Y stable transfected with pre-mir129-1 plasmid compared to control cells trasfected with empty vector before and after differentiation treatment with retinoic acid. As is shown in Figure 9A, the SHSY5Y mir129-1 cell line showed shorter neurites and a bigger and rounded cellular body in compare to control cells. Moreover, the Figure 8A showed the reduced differentiation phenotype following retinoic acid treatment in cells overexpressing mir129-1 with respect to empty vector control. To measure the variance in differentiation level between the cells overexpressing the mir129-1 and the cells with the empty vector we assessed mRNA levels of two genes markers of neuronal differentiation such as ASCL1 and GAD43. We observed, after treatment with retinoic acid, that cells overexpressing miR129-1 showed a lower increase of ASCL1 and GAD43 mRNA levels with respect to control cells after

differentiation (Figure 8B). These results suggest an impairment of neurites formation when mir129-1 is over-expressed.

Figure 8

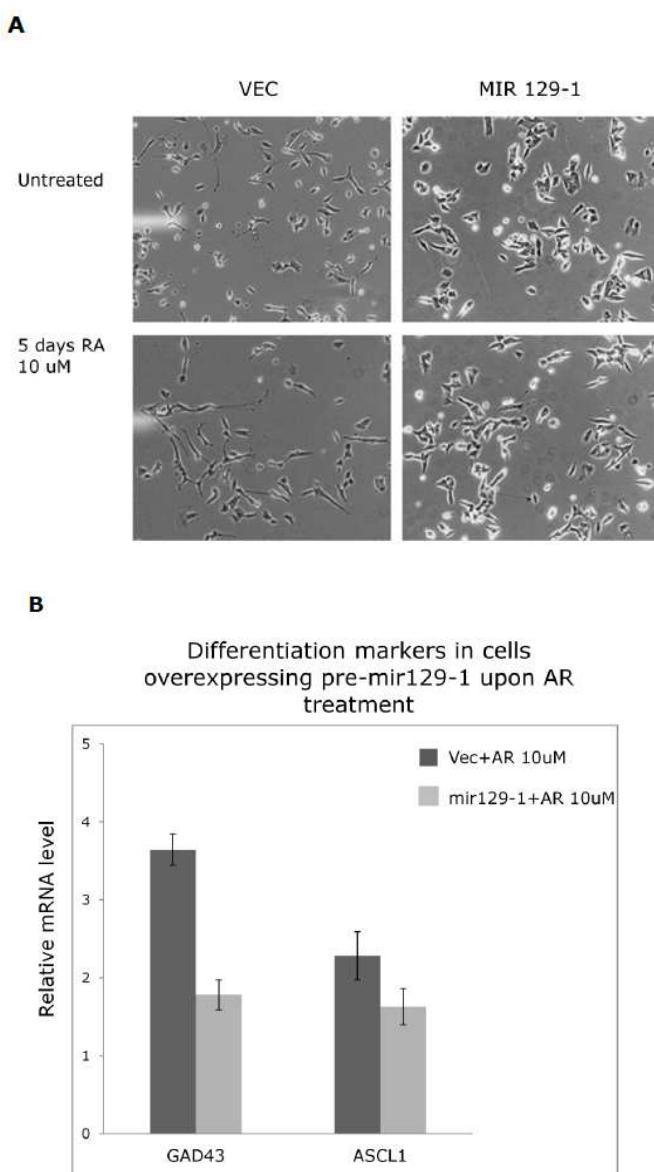


Figure 8: Analysis of the overexpression in SH SY5Y cells. (A) The first lane shows the morphology of the cells transfected with pre-mir129-1 versus cells expressing the empty vector. SHSY5Y overexpressing mir129-1 showed shorter neurites and a bigger and rounded cellular body in compare to control cells (VEC). Upon Retinoic Acid treatment (10uM for 6 days) the VEC cells showed long neurites while only few cells in pre-mir129-1 have an increasing in neurites length. **(B)** Analysis of differentiation level between the cells overexpressing the mir129-1 and the cells with the empty vector. We assessed the mRNA levels of ASCL1 and GAD43 as markers of differentiation. Data were normalized to GAPDH expression.

2.5 Discussion

Post transcriptional regulation of gene expression made by microRNA is increasingly recognized as an important general mechanism by which the function and morphological plasticity of dendrites, axons and synapses are controlled locally in response to various environmental signals. Indeed, there is an increasing number of studies that characterize the expression of miRNAs and their potential role in the molecular mechanisms leading to neurodegenerative diseases. Nevertheless, little is known concerning ALS. Our study started from the observation that miRNA machinery was compromised in a chronic mitochondrial stress and in familial ALS *in vitro* model, the SHSY5Y neuroblastoma cells carrying the SOD1(G93A) mutation. In particular, we found a slight decrease of the two main proteins of microprocessor, Drosha and DGCR8 that could lead to a reduction in the amount of the pre-miRNA cellular pool. The down-regulation of Drosha could be an effect of the observed increase of EWS protein. In fact, it has recently demonstrated that the FET protein member EWS is able to negative regulates Drosha (Kim et al., 2014). On the other hand, the increase in the level of Dicer may result in the processing of a subset of pre-miRs compared to other miRNAs that required different cofactors. Indeed, it has been demonstrated that some Dicer partners proteins are able to bind Dicer and promote the processing of particular miRNAs (Krol et al., 2010). However to elucidate the mechanism by which the expression of specific miRNAs can be modified will be needed further investigations. In this study, we carried out the miRNA profiling by RNA high throughput sequencing in cellular line of a familial SOD1(G93A) ALS model that allowed us to identify a small group of misregulated miRNAs. Only one miRNA, mir-124 in this small pool was already characterized in neurodegenerative disorders (Johnson et al., 2009). More recently mir-200c was linked to synaptic function and neuronal

survival in Huntington disease (Jin et al., 2012). We analysed the dysregulated miRNAs in a small group of ALS mice carrying the same gene mutation at early and symptomatic stage of disease. We found a correlation between mutant SHSY5Y and transgenic SOD1(G93A) mice concerning the up-regulation of mir129-5p and mir200c. As was reasonably expected miRNAs dysregulation in mice differs from the early stage and the end-stage of disease. Intriguingly, we found that the expression of mir129-5p is increased at the early stage while its level decreases with the progression of the disease suggesting that this miR could be one of the first player acting in the pathological cascade that leads to ALS. Thereafter, in order to find a common signature between familial and sporadic ALS and in effort to find new biomarkers in easily accessible sample such as blood, we assessed the two most up-regulated miRNAs in PBMCs obtained from sporadic ALS patients. This would be not the first case in which a microRNA linked to neurodegenerative disorders is found up-regulated both in neuronally differentiated human cells and in human plasma (Gaughwin et al., 2011) candidating it as a biomarker. Subsequently, we studied the predicted targets and we validated ELAVL4/HuD as target of mir129-5p. Recent analysis of ELAVL4/HuD-associated mRNAs in mouse brain revealed that many ELAVL4/HuD targets encoded for proteins with important roles in neuronal differentiation, cytoskeletal transport, and RNA metabolism (Bolognani et al., 2010). These findings fully support the ability of HuD to promote neuronal development, synaptic plasticity, and nerve regeneration (Pascale et al., 2008; Perrone-Bizzozero and Bolognani, 2002). Accordingly, ectopic interventions to overexpress or downregulate HuD in cultured neuronal models revealed a role for HuD in the expression of target mRNAs and in controlling neuronal morphology (Deschênes-Furry et al., 2006). HuD-null mice showed deficient neurogenesis, nerve development, and motor and sensory

functions (Akamatsu et al., 2005). To test the effect of downregulation of HuD due to microRNA regulation, we generated stable cell line overexpressing mir129-5p and we found a reduction in neurite outgrowth and in the expression of differentiation markers with respect to control cells. Taken together these data strongly suggest that microRNAs play a role in ALS pathogenesis and in particular that mir129-5p can affect neuronal plasticity by modulating HuD level. Additionally, it is important to underline that the altered expression of miR129-5p in neuroblastoma SOD1(G93A), in transgenic mice and in sporadic ALS PBMCs, together with the observation that in mice the major increase of miRNA levels is at the early stage, proposes mi129-5p as potential biomarker for ALS. In many patients with ALS in population-based studies, diagnostic certainty currently entails a delay of about 1 year from onset of symptoms to diagnosis (Zoccolella et al., 2006); this delay prevents early treatment with possible disease-modifying drugs. Moreover, at least 30% of anterior horn neurons are thought to have degenerated by the time distal muscle wasting is visible (Swash and Ingram, 1988). Therefore, reliance on symptoms or clinical examination to trigger intervention might not be adequate if degeneration is no longer salvageable at that stage. Thus, an early diagnostic biomarker might prove to be clinically useful only if those at risk of developing ALS can be identified and screened before the onset of symptoms.

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2.6 References

- Abdelmohsen, K., et al., 2010. miR-375 inhibits differentiation of neurites by lowering HuD levels. *Mol Cell Biol.* 30, 4197-210.
- Abelson, J. F., et al., 2005. Sequence variants in SLTRK1 are associated with Tourette's syndrome. *Science.* 310, 317-20.
- Akamatsu, W., et al., 2005. The RNA-binding protein HuD regulates neuronal cell identity and maturation. *Proc Natl Acad Sci U S A.* 102, 4625-30.
- Al-Chalabi, A., et al., 2012. The genetics and neuropathology of amyotrophic lateral sclerosis. *Acta Neuropathol.* 124, 339-52.
- Bartel, D. P., 2009. MicroRNAs: target recognition and regulatory functions. *Cell.* 136, 215-33.
- Beghi, E., et al., 2006. The epidemiology of ALS and the role of population-based registries. *Biochim Biophys Acta.* 1762, 1150-7.
- Bicchi, I., et al., 2013. MicroRNAs and Molecular Mechanisms of Neurodegeneration. *Genes (Basel).* 4, 244-63.
- Bolognani, F., et al., 2010. Novel recognition motifs and biological functions of the RNA-binding protein HuD revealed by genome-wide identification of its targets. *Nucleic Acids Res.* 38, 117-30.
- Butovsky, O., et al., 2012. Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. *J Clin Invest.* 122, 3063-87.
- Chen, L., et al., 2000. Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature.* 408, 936-43.
- Cleveland, D. W., Rothstein, J. D., 2001. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat Rev Neurosci.* 2, 806-19.
- Cuellar, T. L., et al., 2008. Dicer loss in striatal neurons produces behavioral and neuroanatomical phenotypes in the absence of neurodegeneration. *Proc Natl Acad Sci U S A.* 105, 5614-9.
- De Felice, B., et al., 2012. A miRNA signature in leukocytes from sporadic amyotrophic lateral sclerosis. *Gene.* 508, 35-40.
- DeJesus-Hernandez, M., et al., 2011. Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron.* 72, 245-56.
- Deschênes-Furry, J., et al., 2006. The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity. *Bioessays.* 28, 822-33.
- Gaughwin, P. M., et al., 2011. Hsa-miR-34b is a plasma-stable microRNA that is elevated in pre-manifest Huntington's disease. *Hum Mol Genet.* 20, 2225-37.
- Gregory, R. I., et al., 2004. The Microprocessor complex mediates the genesis of microRNAs. *Nature.* 432, 235-40.
- Haramati, S., et al., 2010. miRNA malfunction causes spinal motor neuron disease. *Proc Natl Acad Sci U S A.* 107, 13111-6.
- Hostikka, S. L., et al., 2009. Axial and appendicular skeletal transformations, ligament alterations, and motor neuron loss in Hoxc10 mutants. *Int J Biol Sci.* 5, 397-410.
- Hu, J. Z., et al., 2013. Anti-apoptotic effect of microRNA-21 after contusion spinal cord injury in rats. *J Neurotrauma.* 30, 1349-60.
- Huntzinger, E., Izaurrealde, E., 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet.* 12, 99-110.
- Jin, J., et al., 2012. Interrogation of brain miRNA and mRNA expression profiles reveals a molecular regulatory network that is perturbed by mutant huntingtin. *J Neurochem.* 123, 477-90.
- Johnson, R., et al., 2009. Regulation of neural macroRNAs by the transcriptional repressor REST. *RNA.* 15, 85-96.

- Keller, A., et al., 2011. Toward the blood-borne miRNome of human diseases. *Nat Methods*. 8, 841-3.
- Kim, K. Y., et al., 2014. A multifunctional protein EWS regulates the expression of Drosha and microRNAs. *Cell Death Differ*. 21, 136-45.
- Kim, V. N., et al., 2009. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. 10, 126-39.
- Krol, J., et al., 2010. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet*. 11, 597-610.
- Lee, S. T., et al., 2012. miR-206 regulates brain-derived neurotrophic factor in Alzheimer disease model. *Ann Neurol*. 72, 269-77.
- Lenzken, S. C., et al., 2011. Mutant SOD1 and mitochondrial damage alter expression and splicing of genes controlling neuritogenesis in models of neurodegeneration. *Hum Mutat*. 32, 168-82.
- Liang, C., et al., 2012. MicroRNA-153 negatively regulates the expression of amyloid precursor protein and amyloid precursor-like protein 2. *Brain Res*. 1455, 103-13.
- Liu, C. M., et al., 2013. MicroRNA-138 and SIRT1 form a mutual negative feedback loop to regulate mammalian axon regeneration. *Genes Dev*. 27, 1473-83.
- Majounie, E., et al., 2012. Frequency of the C9orf72 hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study. *Lancet Neurol*. 11, 323-30.
- Packer, A. N., et al., 2008. The bifunctional microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in Huntington's disease. *J Neurosci*. 28, 14341-6.
- Pascale, A., et al., 2008. Defining a neuron: neuronal ELAV proteins. *Cell Mol Life Sci*. 65, 128-40.
- Perrone-Bizzozero, N., Bird, C. W., 2013. Role of HuD in nervous system function and pathology. *Front Biosci (Schol Ed)*. 5, 554-63.
- Perrone-Bizzozero, N., Bolognani, F., 2002. Role of HuD and other RNA-binding proteins in neural development and plasticity. *J Neurosci Res*. 68, 121-6.
- Pratt, A. J., et al., 2012. Amyotrophic lateral sclerosis: update and new developments. *Degener Neurol Neuromuscul Dis*. 2012, 1-14.
- Renton, A. E., et al., 2011. A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron*. 72, 257-68.
- Rosen, D. R., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*. 362, 59-62.
- Schaefer, A., et al., 2007. Cerebellar neurodegeneration in the absence of microRNAs. *J Exp Med*. 204, 1553-8.
- Strickland, I. T., et al., 2011. Axotomy-induced miR-21 promotes axon growth in adult dorsal root ganglion neurons. *PLoS One*. 6, e23423.
- Swash, M., Ingram, D., 1988. Preclinical and subclinical events in motor neuron disease. *J Neurol Neurosurg Psychiatry*. 51, 165-8.
- Volinia, S., et al., 2010. Identification of microRNA activity by Targets' Reverse EXpression. *Bioinformatics*. 26, 91-7.
- Williams, A. H., et al., 2009. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science*. 326, 1549-54.
- Wu, D., Murashov, A. K., 2013. MicroRNA-431 regulates axon regeneration in mature sensory neurons by targeting the Wnt antagonist Kremen1. *Front Mol Neurosci*. 6, 35.
- Yu, Y. M., et al., 2011. MicroRNA miR-133b is essential for functional recovery after spinal cord injury in adult zebrafish. *Eur J Neurosci*. 33, 1587-97.
- Zhou, R., et al., 2009. Evidence for selective microRNAs and their effectors as common long-term targets for the actions of mood stabilizers. *Neuropsychopharmacology*. 34, 1395-405.
- Zoccolella, S., et al., 2006. Predictors of delay in the diagnosis and clinical trial entry of amyotrophic lateral sclerosis patients: a population-based study. *J Neurol Sci*. 250, 45-9.

Chapter 3 -

MEF2D and MEF2C pathways disruption in sporadic and familial ALS patients

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KeyWords: Amyotrophic Lateral Sclerosis (ALS); MEF2D; MEF2C; BDNF; KLF6; RUFY3; NPEPPS; PBMCs.

3.1 Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive neuro-muscular disease characterized by motor neuron loss. MEF2D and MEF2C are members of the myocyte enhancer factor 2 family (MEF2), a group of transcription factors playing crucial roles both in muscle and in neural development and maintenance; for this reason a possible their involvement in ALS context has been investigated. Since the transcriptional activity of each tissue specific MEF2 isoform is conserved in different cell types, we choose to assess our parameters in an easily accessible and widely used experimental tool such as peripheral blood mononuclear cells (PBMCs) obtained from 30 sporadic ALS patients (sALS), 9 ALS patients with mutations in SOD1 gene (SOD1+) and 30 healthy controls. Gene expression analysis showed a significant up-regulation for MEF2D and MEF2C mRNA levels in both sporadic and SOD1+ ALS patients and a direct correlation between MEF2D-MEF2C mRNA levels was observed in patients and controls. Moreover, although protein levels were unchanged, a different pattern of distribution for MEF2D and MEF2C proteins was evidenced by immunohistochemistry, suggesting a possible lack of their function. In line with this hypothesis, a significant down-regulation of the MEF2 downstream targets BDNF, KLF6 and RUFY3 was found. In conclusion our results evidenced, for the first time, a systemic alteration of MEF2D and MEF2C pathways in both sporadic and SOD1+ ALS patients evidencing a possible common feature between the sporadic and the familiar form of disease, potentially eligible as biomarker easily obtainable from accessible biofluids.

3.2 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective degeneration of both upper and lower motor neurons; death usually occurs due to respiratory failure within 3-5 years from symptom onset. Skeletal muscle atrophy and paralysis due to denervation are common features in both patients with spinal or bulbar onset. Causes underlying the pathogenesis of disease remain still unknown, although protein aggregate formation in motor neurons is a neuropathological hallmark (Volkenning et al., 2009). Approximately 90% of patients with amyotrophic lateral sclerosis show a sporadic form (sALS), whereas the remaining 10% have a familial form (fALS); among these, about 20-25% has mutations in CuZn superoxide dismutase (SOD1) gene that catalyzes removal of the superoxide anion radical from cells. However, both sporadic and familial ALS patients display a variety of differing phenotypes (Beghi et al., 2007) making very difficult to define a well-timed and efficient diagnosis and monitoring disease progression; for this reason the validation of biomarkers able to reflect the phenotypic heterogeneity is an urgent goal. These markers should reflect core processes involved in the disease pathogenesis along with great accessibility and low costs in terms of both patient and health system participation.

Recently, Calvo and colleagues (2012) have shown increased myocyte enhancer factor 2-C (MEF2C) mRNA levels in transgenic SOD1^{G93A} mice and similar results were observed after denervation in skeletal muscle cells obtained from wild type mice, highlighting the importance of myogenic potential to compensate muscle damage induced by the neurodegenerative process. MEF2C belongs to the MEF2 protein family, a group of transcriptional regulators including four different isoforms (MEF2A-D) (Yu et al., 1992).

MEF2 family members are highly expressed in cells of muscle lineage where they have been shown to be important regulators during development of skeletal, cardiac and smooth muscle, activating the expression of structural genes, as well as amplifying the expression of other transcription factors that initiate muscle differentiation and maintain sarcomere integrity (Black and Olson, 1998). Furthermore, MEF2 proteins sustain many important functions inside the central nervous system (CNS), where they promote neuronal survival and regulate dendrite morphogenesis, differentiation of post-synaptic structures and excitatory synapse number (Flavell et al., 2006; Mao et al., 1999; Shalizi et al., 2006). MEF2D and MEF2C, in particular, are largely expressed in brain and in muscle where they play crucial roles. Notably, they are directly implicated in the regulation of brain derived neurotrophic factor (BDNF) (Lyons et al., 2012), a neurotrophin already investigated in ALS context due to its ability to promote motor neuron growth and survival in both *in vitro* and *in vivo* models of neuron injury or death (Ikeda et al., 1995; Sendtner et al., 1992), albeit therapeutic trials of BDNF infusion in patients failed to show significant clinical benefit (1999). However, MEF2 proteins are also involved in several functions in circulating immune cells, where they show a specific, conserved expression profile (Swanson et al., 1998). In particular, MEF2C and MEF2D are expressed in lymphocytes and macrophages where they directly sustain the proliferation process and the immune response (Aude-Garcia et al., 2010; Wilker et al., 2008), functions that may be critical for modulating ALS clinical onset and progression. Indeed, immune cells are well known to be directly involved in ALS pathogenesis, actively influencing disease progression and survival in animal models and in patients positively or negatively influencing disease progression in a time-related manner (Henkel et al., 2013; Zhao et al., 2013). Moreover, recent evidences showed that TDP-43

aggregates were able to mediate the activation of microglia causing a toxic pro-inflammatory cascade for motoneurons, suggesting the involvement of a non-cell autonomous pro-inflammatory signalling in motoneuron injury and offering a possible novel therapeutic targets in ALS (Zhao et al., 2015).

Currently, no data exist on the expression and the function of MEF2D and MEF2C in ALS patients. Considering that the transcriptional activity of each tissue-specific MEF2 isoform on the responsive reporter elements is conserved in different cell lines (Sekiyama et al., 2012), we choose to investigate MEF2D and MEF2C gene and protein expression levels in peripheral blood mononuclear cells (PBMCs), an easily accessible and widely used experimental tool able to recapitulate some typical pathological features previously found within CNS of ALS patients, such as TDP-43 aggregates (De Marco et al., 2011; Nardo et al., 2011). Clarifying the involvement of these transcription factors in ALS offers, therefore, the unique opportunity of characterizing a potentially nodal event where disease pathways may converge, since MEF2 mechanism of action is shared by all the cell types involved in ALS pathophysiology. Furthermore, PBMCs offer the opportunity to investigate these parameters using accessible biofluids eligible to be used as source of potential biomarkers useful to be employed in diagnosis and to monitor disease progression.

3.3 Materials and Methods

Recruited subjects

30 patients affected by sporadic ALS (sALS) diagnosed according to El Escorial criteria (Brooks, 2000) were consecutively recruited at the NEMO center (Milano, Italy) following informed consent. ALS patients negative for SOD1, TARDBP and FUS gene mutations were considered eligible for the study and all patients recruited were also assessed for C9orf72 hexanucleotide repeats that were in all subjects <14. Furthermore, 9 familial ALS patients positive for mutations in SOD1 gene (SOD1+) were recruited for the study [SOD1(G93D) n=6; SOD1(L144F) n=1; SOD1(D90A) n=1; SOD1(G72S) n=1]. See Table 1 for demographic and clinical characteristics. ALSFRS-R scores were recorded as well as the disease progression index (DPI), defined as: [(48 - ALSFRS-R score at recruitment)/disease duration in months](Tremolizzo et al., 2013). 30 healthy subjects, age- and sex-matched to sALS patients, were recruited. Exclusion criteria for all the recruited subjects were considered cancer, autoimmune and inflammatory diseases. Moreover, healthy controls were not affected by any neurological or psychiatric condition, nor were taking psychoactive drugs. This study was approved by *Ethics Committee* of University of Milan-Bicocca (Protocol n. 0017525/15; 04 August 2015).

	CTRL n=30	sALS n=30	SOD1+ n=9
Gender, M/F	16 / 14	16 / 14	3 / 6
Age, yr	61.6 ± 10.6 (37-77)	62.6 ± 9.9 (41-78)	58.5 ± 11.8 (40-78)
Duration, mo	N/A	32.9 ± 24.5 (3-82)	39.0 ± 34.6 (0-96)
Onset, B/S	N/A	9 / 21	0 / 9
ALSFRS-R	N/A	24.1 ± 10.2 (6-44)	24.0 ± 20.3 (4-47)
DPI	N/A	0.92 ± 0.51 (0.45- 2.33)	0.71 ± 0.54 (0.22- 1.63)
PEG, yes/no	N/A	8 / 22	4 / 5
NIV, yes/no	N/A	11 / 19	2 / 7
Riluzole, yes/no	N/A	26 / 4	6 / 3

Table 1

Clinical and demographic characteristics of the recruited subjects. Data are presented as mean ± standard deviation (range). ALSFRS-R, ALS functional rating scale-revised version; CTRL, healthy controls; DPI, disease progression index; N/A, not applicable; NIV, non-invasive ventilation; PEG, percutaneous endoscopic gastrostomy; sALS, sporadic ALS; SOD1+, ALS patients positive for SOD1 gene mutations.

PBMCs isolation

Peripheral blood samples (15 ml) were drawn from each subject in tubes containing K₂EDTA after overnight fasting. PBMCs were collected as previously described (Sala et al., 2012). Briefly, whole blood samples were diluted with the same amount of saline solution, layered on Ficoll-Histopaque (GE Healthcare) and centrifuged (490×g, 30 min, room temperature). PBMCs were collected from the interface between plasma and Ficoll-Histopaque, washed with saline solution, aliquoted and stored at -80 °C.

RNA extraction and cDNA synthesis

Total RNA was extracted from PBMCs using the RNeasy Mini kit (Qiagen), according to the manufacturer instructions. RNA concentration was determined spectrophotometrically at 260 nm and each RNA sample was loaded on 2% agarose gel to evaluate RNA quality. cDNA was synthesized from RNA using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) at the following conditions: 10 min at 25 °C and 60 min at 42 °C. The reaction was terminated at 85 °C for 5 min and cDNAs stored at -20 °C.

Real-time PCR (qPCR)

For the analysis of MEF2D and MEF2C mRNA levels, cDNAs obtained from 70 ng RNA were amplified in quadruplicate in the ABI Prism 7500 HTSequence Detection System (Applied Biosystems) using TaqMan® Gene Expression Assay (Applied Biosystems, MEF2D assay ID: Hs00954735_m1; MEF2C assay ID: Hs00231149_m1; beta-actin assay ID: Hs99999903_m1). For the analysis of mRNA levels of the remaining targets, cDNAs obtained from 50 ng RNA were amplified in triplicate using the HOT FIREPol®

EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne) at the following conditions: 50 °C for 2 min, 95 °C for 15 min, 40 cycles of: 95 °C for 15 s, 60 °C for 20 s and 72 °C for 30 s. The following primer pairs (Sigma-Aldrich) were used:

BDNF-F (TGGCTGACACTTCGAACAC)

BDNF-R (AGAAGAGGAGGCTCCAAAGG);

KLF6-F (GGACCAAATTCTAGCTCGGG)

KLF6-R (AGGCGTCGCCATTACCCTTG);

RUFY3-F (CCTGGGAATTGCTTGCCCTA)

RUFY3-R (TCTCATAGGATTGGCCGTGC);

NPEPPS-F (CGACTTGCTGGACTTCACCTT)

NPEPPS-R (CTGATTAGTCGCCTGCCTCAC);

beta-actin-F (TGTGGCATCCACGAAACTAC)

beta-actin-R (GGAGCAATGATCTTGATCTCA).

For relative quantification of each target vs. beta-actin mRNA, the comparative Ct method was used as previously described (Sala et al., 2010).

Western blotting

Cell pellets were lysed in cell extraction buffer (Invitrogen) supplemented with 1 mM PMSF (Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich) and protein concentrations determined by Bradford's method. For each target, preliminary experiments were run loading on blot increasing amounts of total proteins obtained from a pool of three control PBMCs to verify the linearity of the immunoreactive signals and

to choose the most suitable protein amount for the semi-quantification. Based on these results, 40 µg of proteins were diluted in Laemmli's loading buffer pH 6.8, denatured at 95°C for 2 min, separated by SDS-PAGE in 4-12% tris glycine gels (Invitrogen) and transferred to nitrocellulose. Blots were blocked for 1 h, incubated overnight at 4°C with specific primary antibodies (MEF2D, BD Biosciences, 1:1,000 dilution; MEF2C, Cell Signaling, 1:500 dilution, respectively) and then with HRP-linked anti-mouse or -rabbit IgG for 1 h. Beta-actin (Sigma-Aldrich, 1:40,000 dilution) was used as internal standard. Signals were revealed by chemi-luminescence, visualized on X-ray film and quantified by an imaging densitometer (Bio-Rad). The protein expression of each sample was expressed as ratio between the optical densities of the target protein and beta-actin. Negative control blots, obtained incubating membranes without the primary antibody and in presence of the HRP- linked anti-mouse or anti-rabbit IgG, were run before starting the quantification and no immunoreactive signal was observed. Four ALS samples, four control samples (comparable for age and sex to ALS samples), a molecular weight marker (GE Healthcare) and a quality control sample were analyzed in each blot. Each value was calculated referred to the mean value of the controls loaded on each gel. For all blots, quality control sample was represented by total protein extracts obtained from SH-SY5Y human neuroblastoma cells. Each sample was analyzed in duplicate in 2 different blots and the deriving coefficient of variation (CV), calculated after inter-membrane normalization, was always <15%.

Immunofluorescence

PBMC smears were fixed with 4% paraformaldehyde for 15 min and the permeabilized using 0.2% Triton X-100 for 10 min. Cells were incubated overnight at 4 °C with anti-

MEF2D primary antibody (1:100, mouse anti-MEF2D, BD Biosciences) or anti-MEF2C primary antibody (1:50, rabbit anti-MEF2C, Cell Signaling) and then with appropriate fluorochrome-conjugated secondary antibody diluted in PBS (1:200, Alexa Fluor 488 goat anti-mouse, Invitrogen, and 1:200, Alexa Flour 488 goat anti-rabbit, Invitrogen) for 1 hour. After staining with DAPI for 2 min (1:50000, Sigma-Aldrich), glass slides were mounted with polyvinyl alcohol (Polyvinyl Alcohol mounting medium with DABCO® Antifading pH 8.7, Sigma-Aldrich). Immunofluorescence analysis was performed with laser confocal microscope (Radiance 2100; Biorad Laboratories).

Statistical analysis

Statistical analysis was performed using Prism 4.00 (GraphPad Software). Data are expressed throughout the paper as mean \pm standard deviation (SD). Two-tailed unpaired Student's *t*-test or one-way ANOVA, followed by Tukey's multiple comparison test, were used to assess the significance of differences between two or more groups, respectively. Correlation was computed with the two tailed Pearson's *r*-test. Values that differ by two standard deviations from the mean value of each group were excluded from the analysis.

3.4 Results

Up-regulation of MEF2D and MEF2C mRNA levels in sALS and SOD1+ PBMCs

A significant increase in both MEF2D and MEF2C mRNA levels was evidenced in PBMCs of sporadic and SOD1+ ALS patients with respect to controls (**Figure 1A** and **1B**). MEF2D mRNA levels of sporadic patients were more than two-fold higher ($p<0.001$) as compared to healthy controls; SOD1+ ALS patients showed increased MEF2D mRNA levels with respect to both sALS patients (+73%, $p<0.001$) and controls (+342%, $p<0.001$) (**Figure 1A**). Similar results were obtained for MEF2C mRNA levels that were significantly up-regulated (three-fold, $p<0.001$) in both sporadic and SOD1+ ALS patients when compared to controls (**Figure 1B**). A positive correlation between mRNA levels of MEF2D and MEF2C was observed in both healthy controls ($r=0.43$, $p<0.05$), sALS ($r=0.43$, $p<0.05$) and SOD1+ patients ($r= 0.89$, $p<0.01$) (**Figure 2**), suggesting the existence of a common regulatory mechanism responsible for the transcription of these MEF2 isoforms.

Figure 1

MEF2D and MEF2C gene expression in PBMCs of patients and healthy controls. Increased MEF2D (**A**) and MEF2C (**B**) mRNA levels in PBMCs of sporadic ALS patients (sALS) and subjects with SOD1 gene mutations (SOD1+) with respect to healthy controls (CTRLs). Relative quantification (RQ) of MEF2D and MEF2C mRNA is calculated as ratio to β -actin. One-way ANOVA, followed by Tukey's multiple comparison test (**p<0.001) (CTRLs n=30; sALS n=30; SOD1+ n=9).

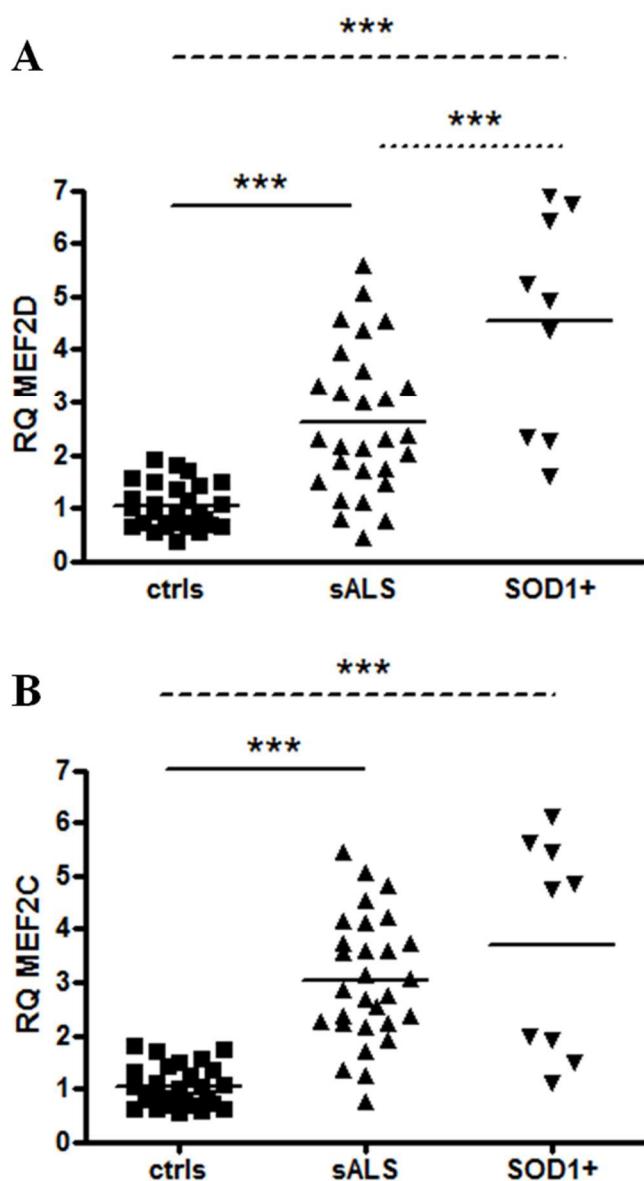
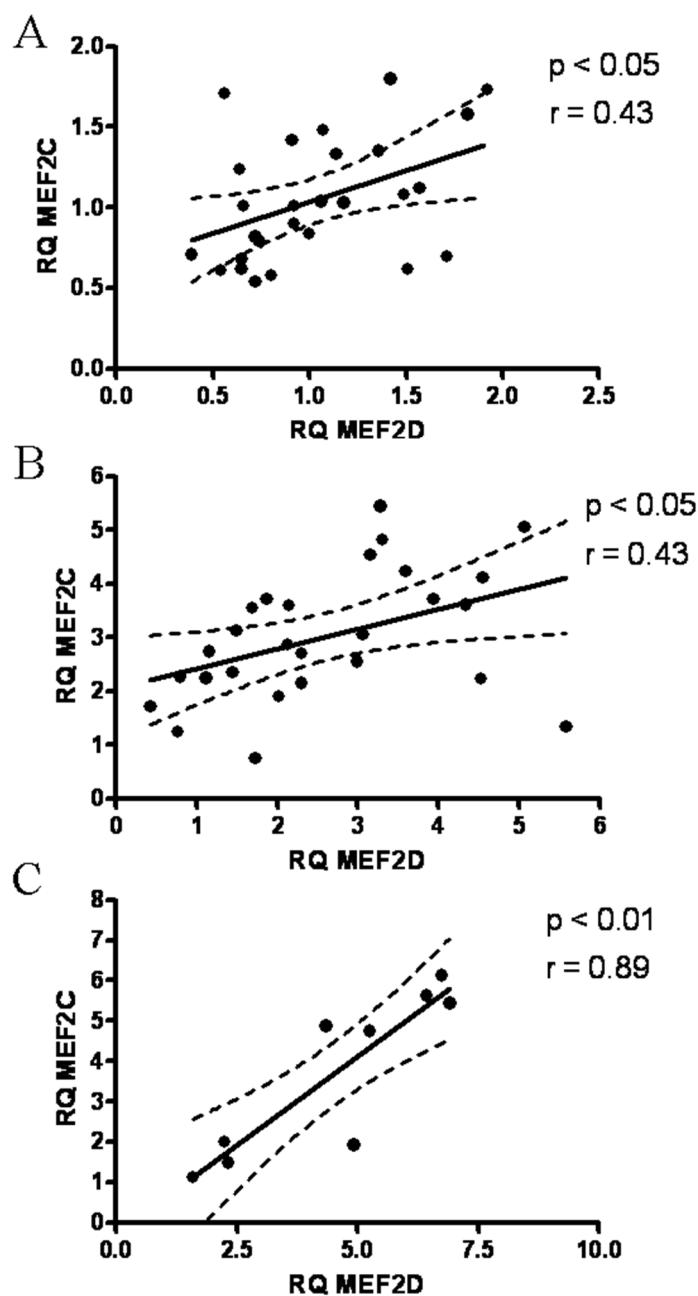


Figure 2

Correlation between mRNA levels of different MEF2 isoforms. MEF2D and MEF2C mRNA levels showed a positive correlation in PBMCs of healthy controls (**A**. $p<0.05$; $r=0.43$; $n=30$), sporadic ALS patients (**B**. $p<0.05$; $r=0.43$; $n=30$) and SOD1+ patients (**C**. $p<0.01$; $r=0.89$; $n=9$).



Different localization of MEF2D-MEF2C in PBMCs of sporadic ALS patients

Protein expression analyses performed by Western blotting on PBMCs lysates evidenced a remarkable individual variability for both MEF2D and MEF2C targets; with a subgroup of subjects showing undetectable MEF2D (14 patients and 13 controls) and MEF2C (12 sALS and 11 controls) protein levels, as previously reported (Sala et al., 2014). Collectively, MEF2D and MEF2C immunoreactive levels were unchanged in sALS with respect to controls (**Figure 3**). Based on the hypothesis that an altered subcellular distribution might occur without changing the overall levels, an immunofluorescence assay was performed on fixed PBMCs derived from three sALS patients and three healthy controls. Different patterns of distribution were observed for both MEF2D and MEF2C immunoreactive signals. In PBMCs obtained from healthy controls MEF2D staining marked well-defined speckles within the nucleus, while in sALS patients the signal was more diffused (**Figure 4A and 4B**). On the other hand, MEF2C immunoreactive signal, albeit qualitatively more similar between patients and controls, was found more frequently outside the nuclei (**Figure 4C and 4D**), bordering them, in sALS PBMCs (~8%) with respect to healthy controls.

Figure 3

MEF2D and MEF2C protein expression is similar in PBMCs of sALS patients and controls (CTRL). Individual MEF2D (A) and MEF2C (B) protein levels in PBMC lysates obtained from 30 sALS patients and 30 healthy controls (CTRL), assessed by Western blotting. MEF2D and MEF2C protein levels are expressed as ratio between target and beta-actin optical density, each value was calculated referred to the mean value of the controls loaded on each gel. Representative Western blot image (C) showing MEF2D and MEF2C protein levels in sALS (n=4) with respect to controls (n=4).

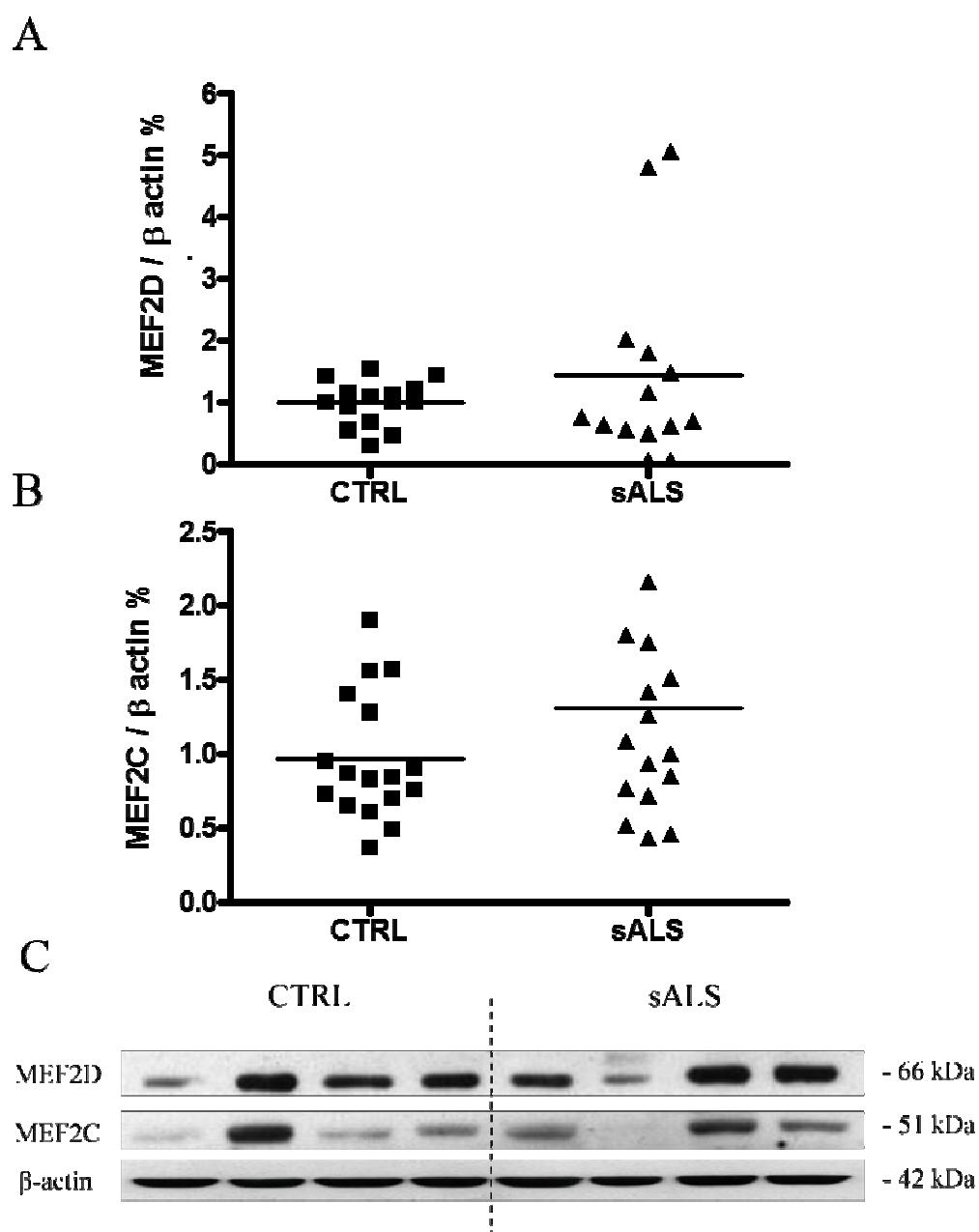
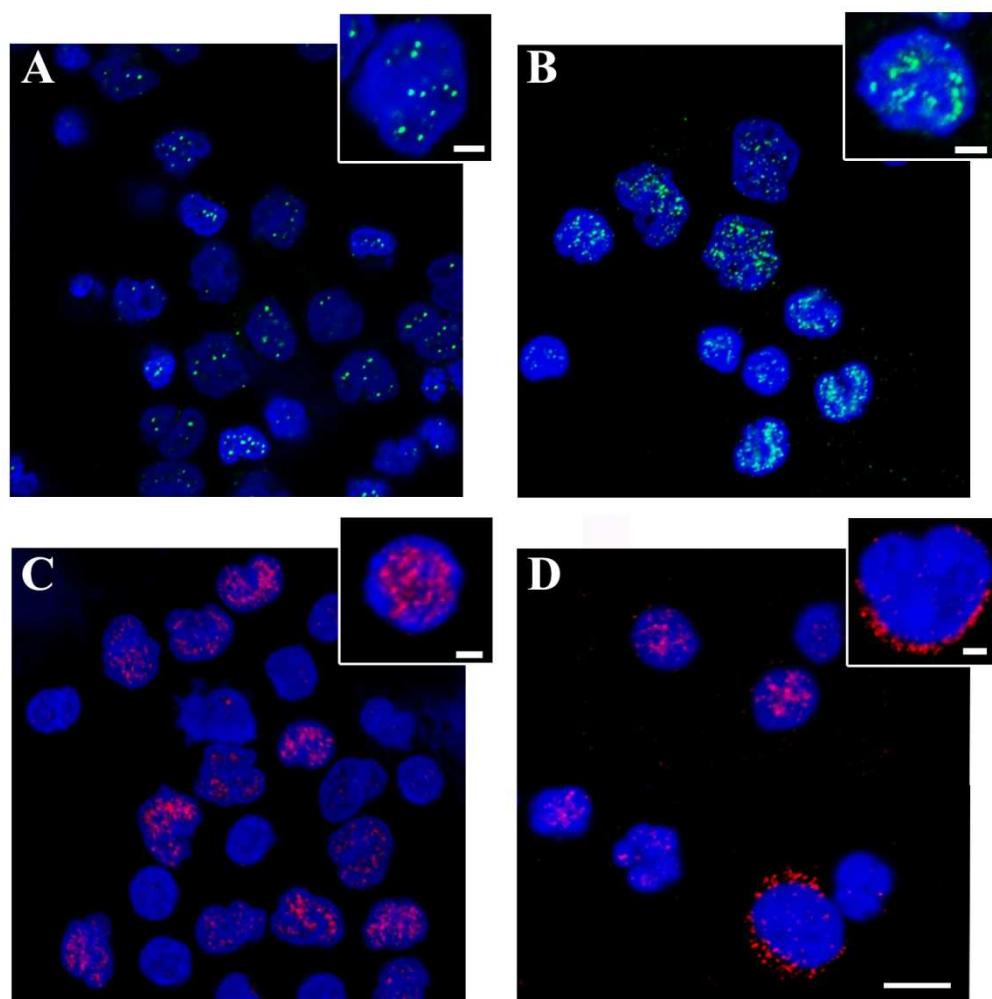


Figure 4

Different MEF2D and MEF2C localization in sALS PBMCs with respect to controls. Representative immunofluorescent assay showing MEF2D (A,B, green fluorescence) and MEF2C (C,D, red fluorescence) expression in PBMCs of 3 healthy controls (A,C) and 3 sALS patients (B,D). Nuclei are stained in blue (DAPI). Insert bar = 10 μ m; insert bar in the box = 2 μ m



Down-regulation of MEF2D-MEF2C downstream targets in PBMCs of sporadic and SOD1+ ALS patients

To verify if the normal function of MEF2D and MEF2C was influenced by the suggested altered localization observed in immunofluorescence, a quantification of selected downstream targets of these transcription factors was performed. BDNF mRNA levels, whose transcription process is regulated by both MEF2D and MEF2C isoforms (Lyons et al., 2012), were found to be significantly reduced in both sALS (-42%, p<0.05) and SOD1+ patients (-95%, p<0.01) when compared to healthy controls (**Figure 5A**). Considering that BDNF regulation involves other players besides MEF2D and MEF2C, we decided to include in our analysis selected downstream targets that are specifically regulated by MEF2D or MEF2C. Krueppel-like factor 6 (KLF6), a specific MEF2D-target (Salma and McDermott, 2012), was found to be down-regulated in sALS patients (-46%, p<0.0001) and in SOD1+ subjects (-49%, p<0.01) with respect to controls (**Figure 5B**). Furthermore, the expression analysis of RUN and FYVE domain containing 3 (RUFY3), a MEF2C target (Sekiyama et al., 2012), showed a significant reduction in mRNA levels in both sporadic (-53%, p<0.05) and SOD1+ (-65%, p<0.05) patients versus controls (**Figure 5C**). In contrast, the analysis of mRNA levels of puromycin-sensitive aminopeptidase (NPEPPS), another MEF2C specific target, showed no differences between patients and controls (**Figure 5D**).

A positive correlation was found between BDNF mRNA levels and the specific MEF2D-target KLF6 both in controls ($r= 0.54$, p<0.01) and in sALS patients ($r= 0.51$, p<0.01) (**Figure 6A-6C**) but not in SOD1+ subjects. BDNF mRNA levels instead correlated with the MEF2C downstream target RUFY3 in healthy controls ($r= 0.69$, p<0.05) and in SOD1+ subjects ($r= 0.75$, p<0.05) (**Figure 6B-6D**) but not in sALS.

Figure 5

MEF2D and MEF2C downstream targets mRNA levels in PBMCs of the recruited subjects. Relative quantification (RQ) of target mRNA is calculated as ratio to β -actin. Both BDNF (**A**), KLF6 (**B**), and RUFY3 (**C**) are down-regulated in sALS and SOD1+ patients with respect to healthy controls (CTRL), while no change was observed in NPEPPS gene expression (**D**). One-way ANOVA, followed by Tukey's multiple comparison test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) (CTRLs n=30; sALS n=30; SOD1+ n=9).

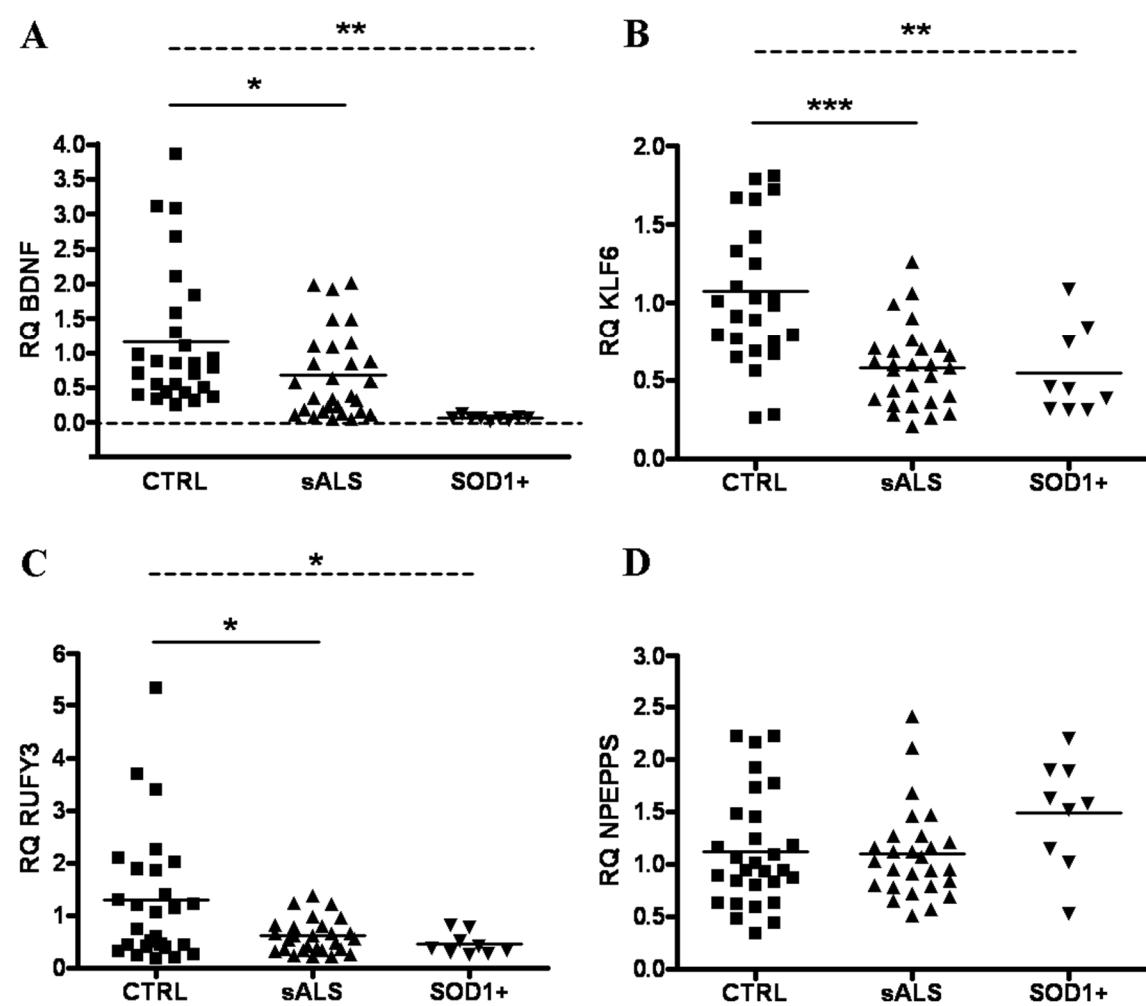
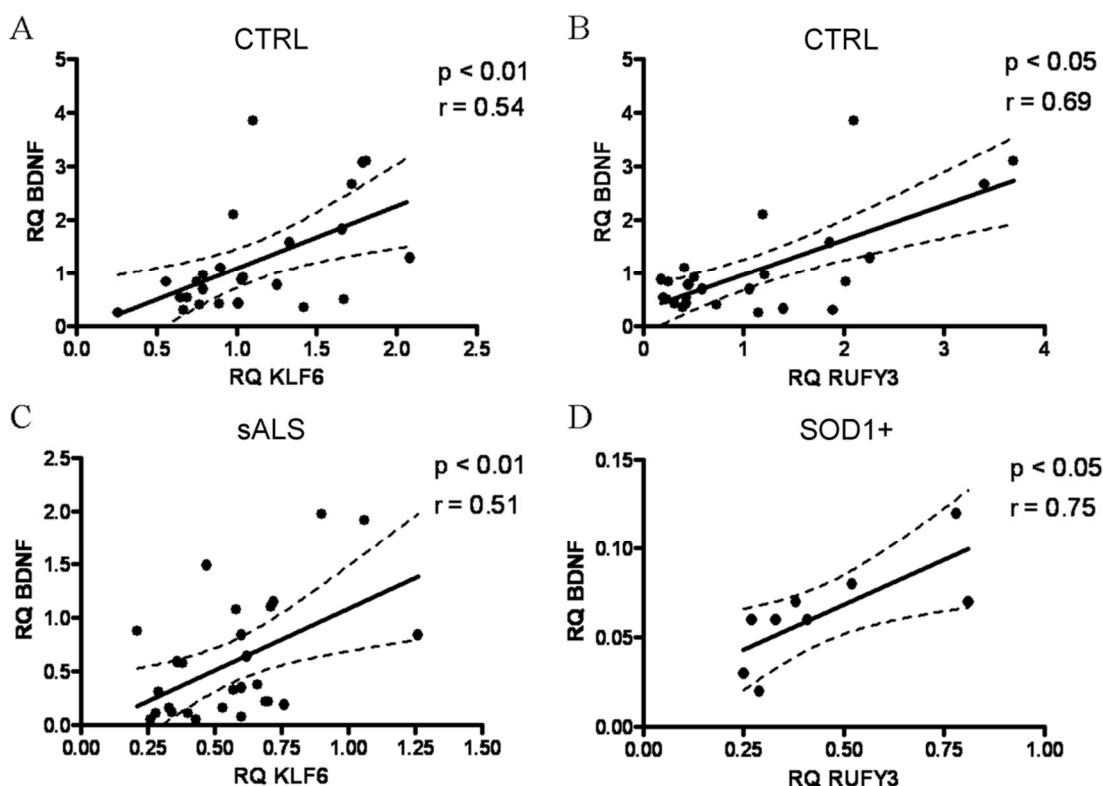


Figure 6

Correlation between BDNF and the specific MEF2D-downstream target KLF6 mRNA levels in healthy controls (**A**; $r=0.54$, $p<0.01$, $n=30$) and in sALS patients (**C**; $r=0.51$, $p<0.01$, $n=30$). BDNF mRNA levels also correlate with the specific MEF2C-downstream target RUFY3 mRNA levels in controls (**B**; $r= 0.69$, $p<0.05$, $n=30$) and in SOD1+ ALS patients (**D**; $r= 0.75$, $p<0.05$, $n=9$).



No correlations emerged between biological parameters and clinical characteristics

Dichotomizing ALS patients based on riluzole assumption (5 untreated vs. 25 treated sALS patients; 3 untreated vs. 6 treated SOD1+ ALS patients), no significant differences emerged. No influence of other clinical characteristics (sex, age at onset, site of onset, disease duration, ALSFRS-R score and disease progression index) of patients was detected on all investigated parameters.

3.5 Discussion

MEF2 transcription factors play pivotal functions in brain, skeletal muscles and PBMCs, regulating the synthesis of specific mRNA targets through common mechanisms conserved in all these different cell lines (Sekiyama et al., 2012). Here we report a strong up-regulation of MEF2D and MEF2C mRNA levels in PBMCs obtained from sporadic and SOD1+ ALS patients when compared to healthy matched controls; furthermore, a different pattern of distribution of the related immunofluorescence signal was also observed in sALS. Based on these evidences, a possible lack of function for MEF2D and MEF2C in ALS patients has been hypothesized. To evaluate MEF2D transcriptional activity we assessed mRNA levels of Krueppel-like factor 6 (KLF6), a MEF2D downstream target essential for its pro-survival function. Indeed, knockdown of KLF6 expression in neurons promotes neuronal cell death, antagonizing the beneficial effect of MEF2D (Salma and McDermott, 2012). Similarly, MEF2C transcriptional activity was evaluated by assessing mRNA levels of RUN and FYVE domain containing 3 (RUFY3) and puromycin-sensitive aminopeptidase (NPEPPS), two MEF2C downstream targets (Sekiyama et al., 2012). Our results showed a significant reduction of KLF6 and RUFY3 mRNA levels in both sporadic and SOD1+ ALS patients, confirming the hypothesis of a reduced transcriptional activity of MEF2D and MEF2C. To further confirm these results, the expression of brain derived neurotrophic factor (BDNF), another target regulated by MEF2 proteins, was evaluated. Since MEF2D and MEF2C differently regulate BDNF acting on distinct alternative promoters (Lyons et al., 2012), the quantification of mRNA levels was performed using specific primers designed to amplify all the most important BDNF transcript variants (specific for 17 out of total 18 alternative transcripts). We

demonstrated a significant down-regulation of BDNF gene expression in both sALS and SOD1+ subjects and a positive correlation between BDNF mRNA levels and mRNA levels of KLF6 and RUFY3 both in patients and controls was also observed, suggesting that BDNF expression is directly linked to activity of MEF2D and MEF2C proteins. Therefore, although the regulation of BDNF-MEF2 is very complex and only partially clarified (Lyons et al., 2012; Pazyra-Murphy et al., 2009; Wang et al., 2007), it is possible to hypothesize that the significant reduction in BDNF synthesis that we found in ALS patients is probably due to a synergic reduction in the activity of both MEF2 isoforms. Although the level of the MEF2D and MEF2C proteins were unchanged, a strong up-regulation of their mRNA levels was observed in PBMCs of both sporadic and SOD1+ ALS patients, confirming previous results obtained in SOD1^{G93A} ALS animal model (Calvo et al., 2012). We hypothesize that the increased transcription of the MEF2 genes observed in this study may represent a compensatory effect of the MEF2 lack of function, indicated by the decreased mRNA levels of downstream targets, suggesting an autoregulatory feedback loop. Similar results were previously observed in human neuroblastoma cells where a significant increase of MEF2D mRNA levels was shown in response to a toxic agent (Sala et al., 2013). Interestingly, our results showed a direct correlation between MEF2D and MEF2C mRNA levels conserved in sALS patients, in SOD1+ subjects as well as in healthy controls, suggesting the existence of an upstream regulatory mechanism common to both factors, which is unaltered in disease conditions. Our data suggest that the pathological MEF2D/MEF2C loss of function, which is reflected by the reduction of the expression of their downstream targets, is not caused by changes in mRNA synthesis or translation, but it is likely due to modifications of mature MEF2 proteins. MEF2 function is finely regulated by many different post-transcriptional

modifications and interaction with several activators or repressive elements (Gong et al., 2003; McKinsey et al., 2001). Among these, members of class IIa histone deacetylases (HDACs) play a very important role in the regulation of MEF2 protein activity; indeed, in absence of stimuli, class-II HDACs maintain MEF2 in a repressive state until transactivating stimuli arrive. In particular, HDAC4 has been shown to promote the sumoylation of the transcriptional domain of MEF2D and MEF2C, repressing their activity and regulating important cellular programs like muscle differentiation, neuronal survival and T-cell apoptosis (Cohen et al., 2009; Grégoire et al., 2006; Grégoire and Yang, 2005). Interestingly, HDAC4 levels were found to be dramatically increased in muscles of SOD1^{G93A} animal model, with subsequent repression of MEF2 target genes, contributing to muscle dysfunction (Cohen et al., 2009; Cohen et al., 2007). In this context, a treatment with HDAC inhibitors has been shown to alleviate the disease phenotype in ALS mouse model (Ryu et al., 2005). Furthermore, a crucial role for HDAC4 and its regulator microRNA-206 (miR-206) in ALS progression was recently suggested; as a matter of fact, miR-206, a skeletal muscle-specific microRNA, was shown to promote reinnervation and slow down disease progression in ALS mice, repressing muscle expression of HDAC4 thus counteracting the negative effect of HDAC4 on reinnervation (Williams et al., 2009). Finally, in a recent study performed in muscle samples from ALS patients, HDAC4 expression was significantly higher in patients with rapidly progressive disease and was negatively correlated with the extent of muscle reinnervation and functional outcome (Bruneteau et al., 2013). Moreover, 14-3-3 proteins, which are involved in MEF2 regulation by disrupting its interaction with class-II HDACs, have been found to be accumulated in the characteristic ubiquitinylated inclusions present in the spinal cord of ALS patients (McKinsey et al., 2000; Volkening

et al., 2009), thus contributing to MEF2 repression by HDACs. These data are in line with the part of the literature describing alterations of chromatin methylation and acetylation status in ALS (Liu et al., 2013; Tremolizzo et al., 2014), thus reinforcing the involvement of epigenetic modifications in the pathogenesis of disease. Taken together, these evidences allow us to suppose that an imbalance in MEF2-HDAC ratio demonstrated in ALS could determine a loss of MEF2D and MEF2C function, with consequent disruption of many critical processes in both muscle, neuronal and immune system cells.

3.6 Conclusions

This study indicates that a dysfunction of the MEF2 pathway play a potential pathogenic role in ALS. Besides MEF2D and MEF2C, also their downstream targets are known to be crucial for many cellular functions in both the nervous system and skeletal muscle. KLF6 regulates neuronal cell survival and axon growth as well as skeletal myogenesis (Dionyssiou et al., 2013; Salma and McDermott, 2012); RUFY3/Singar1 can suppress the formation of surplus axons and contribute to neural polarity (Mori et al., 2007) and NPEPPS is an aminopeptidase that counteracts the accumulation of aggregation-prone proteins and thus proteotoxicity in brain (Menzies et al., 2010). NPEPPS directly regulates SOD1 protein abundance and clearance via proteolysis and, differently from results of our study, NPEPPS protein expression was observed markedly decreased in transgenic mice overexpressing mutant SOD1 and in postmortem spinal cord of sporadic ALS patients (Ren et al., 2011). Also BDNF has been previously investigated in ALS field, although the literature regarding BDNF expression in peripheral tissues or in affected muscles of ALS patients is discordant and might deserve further characterization (Grundström et al., 2000; Küst et al., 2002).

In conclusion, our results support the hypothesis that MEF2D, MEF2C and their downstream targets found altered in ALS patients, might be involved in the pathogenesis of disease. Moreover, alterations identified in MEF2C and MEF2D pathways were observed in both sporadic and SOD1+ ALS patients. Since sporadic and familial forms of disease have completely different pathological hallmarks, it could be very important to identify a common set of altered parameters because pathogenic mechanisms

sustaining these alterations probably occur in a crucial moment of disease onset and represent a common feature of both pathological forms. Furthermore, our findings evidenced a different expression of assessed parameters between ALS patients and controls using accessible biofluids, offering the opportunity to explore this tool in future to better understand if could be employed to development a suitable diagnostic tool. Indeed, our preliminary data seem to suggest that alterations in MEF2 pathways could be associated with a different disease progression index (DPI) (data not shown). To address this important issue a dedicated study will be performed following up patients in different time points to monitor our parameters during disease progression.

Moreover, the identification of MEF2 pathway as novel factor potentially involved in ALS pathogenesis suggests that further investigations could be justified in affected tissues obtained from patients or in motor neurons and skeletal muscles deriving from ALS animal models. In fact, the confirmation of MEF2 pathway disruption in ALS might lead to elucidate the possible underlying causes, offering novel clues for dedicated therapeutic interventions.

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3.7 References

1999. A controlled trial of recombinant methionyl human BDNF in ALS: The BDNF Study Group (Phase III). *Neurology*. 52, 1427-33.
- Aude-Garcia, C., et al., 2010. Dual roles for MEF2A and MEF2D during human macrophage terminal differentiation and c-Jun expression. *Biochem J*. 430, 237-44.
- Beghi, E., et al., 2007. The heterogeneity of amyotrophic lateral sclerosis: a possible explanation of treatment failure. *Curr Med Chem*. 14, 3185-200.
- Black, B. L., Olson, E. N., 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol*. 14, 167-96.
- Brooks, B. R., 2000. Problems in shortening the time to confirmation of ALS diagnosis: lessons from the 1st Consensus Conference, Chicago, May 1998. *Amyotroph Lateral Scler Other Motor Neuron Disord*. 1 Suppl 1, S3-7.
- Bruneteau, G., et al., 2013. Muscle histone deacetylase 4 upregulation in amyotrophic lateral sclerosis: potential role in reinnervation ability and disease progression. *Brain*. 136, 2359-68.
- Calvo, A. C., et al., 2012. Genetic biomarkers for ALS disease in transgenic SOD1(G93A) mice. *PLoS One*. 7, e32632.
- Cohen, T. J., et al., 2009. The deacetylase HDAC4 controls myocyte enhancing factor-2-dependent structural gene expression in response to neural activity. *FASEB J*. 23, 99-106.
- Cohen, T. J., et al., 2007. The histone deacetylase HDAC4 connects neural activity to muscle transcriptional reprogramming. *J Biol Chem*. 282, 33752-9.
- De Marco, G., et al., 2011. Cytoplasmic accumulation of TDP-43 in circulating lymphomonocytes of ALS patients with and without TARDBP mutations. *Acta Neuropathol*. 121, 611-22.
- Dionyssiou, M. G., et al., 2013. Krüppel-like factor 6 (KLF6) promotes cell proliferation in skeletal myoblasts in response to TGF β /Smad3 signaling. *Skelet Muscle*. 3, 7.
- Flavell, S. W., et al., 2006. Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. *Science*. 311, 1008-12.
- Gong, X., et al., 2003. Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. *Neuron*. 38, 33-46.
- Grundström, E., et al., 2000. GDNF but not BDNF is increased in cerebrospinal fluid in amyotrophic lateral sclerosis. *Neuroreport*. 11, 1781-3.
- Grégoire, S., et al., 2006. Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. *J Biol Chem*. 281, 4423-33.
- Grégoire, S., Yang, X. J., 2005. Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. *Mol Cell Biol*. 25, 2273-87.
- Henkel, J. S., et al., 2013. Regulatory T-lymphocytes mediate amyotrophic lateral sclerosis progression and survival. *EMBO Mol Med*. 5, 64-79.
- Ikeda, K., et al., 1995. Effects of brain-derived neurotrophic factor on motor dysfunction in wobbler mouse motor neuron disease. *Ann Neurol*. 37, 505-11.
- Küst, B. M., et al., 2002. Elevated levels of neurotrophins in human biceps brachii tissue of amyotrophic lateral sclerosis. *Exp Neurol*. 177, 419-27.
- Liu, D., et al., 2013. Proteomic analysis reveals differentially regulated protein acetylation in human amyotrophic lateral sclerosis spinal cord. *PLoS One*. 8, e80779.
- Lyons, M. R., et al., 2012. Members of the myocyte enhancer factor 2 transcription factor family differentially regulate Bdnf transcription in response to neuronal depolarization. *J Neurosci*. 32, 12780-5.
- Mao, Z., et al., 1999. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. *Science*. 286, 785-90.

- McKinsey, T. A., et al., 2000. Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. *Proc Natl Acad Sci U S A.* 97, 14400-5.
- McKinsey, T. A., et al., 2001. Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev.* 11, 497-504.
- Menzies, F. M., et al., 2010. Puromycin-sensitive aminopeptidase protects against aggregation-prone proteins via autophagy. *Hum Mol Genet.* 19, 4573-86.
- Mori, T., et al., 2007. Singar1, a novel RUN domain-containing protein, suppresses formation of surplus axons for neuronal polarity. *J Biol Chem.* 282, 19884-93.
- Nardo, G., et al., 2011. Amyotrophic lateral sclerosis multiprotein biomarkers in peripheral blood mononuclear cells. *PLoS One.* 6, e25545.
- Pazyra-Murphy, M. F., et al., 2009. A retrograde neuronal survival response: target-derived neurotrophins regulate MEF2D and bcl-w. *J Neurosci.* 29, 6700-9.
- Ren, G., et al., 2011. Cu, Zn-superoxide dismutase 1 (SOD1) is a novel target of Puromycin-sensitive aminopeptidase (PSA/NPEPPS): PSA/NPEPPS is a possible modifier of amyotrophic lateral sclerosis. *Mol Neurodegener.* 6, 29.
- Ryu, H., et al., 2005. Sodium phenylbutyrate prolongs survival and regulates expression of anti-apoptotic genes in transgenic amyotrophic lateral sclerosis mice. *J Neurochem.* 93, 1087-98.
- Sala, G., et al., 2013. Rotenone upregulates alpha-synuclein and myocyte enhancer factor 2D independently from lysosomal degradation inhibition. *Biomed Res Int.* 2013, 846725.
- Sala, G., et al., 2010. Vesicular monoamine transporter 2 mRNA levels are reduced in platelets from patients with Parkinson's disease. *J Neural Transm.* 117, 1093-8.
- Sala, G., et al., 2014. Reduced expression of the chaperone-mediated autophagy carrier hsc70 protein in lymphomonocytes of patients with Parkinson's disease. *Brain Res.* 1546, 46-52.
- Sala, G., et al., 2012. A panel of macroautophagy markers in lymphomonocytes of patients with amyotrophic lateral sclerosis. *Amyotroph Lateral Scler.* 13, 119-24.
- Salma, J., McDermott, J. C., 2012. Suppression of a MEF2-KLF6 survival pathway by PKA signaling promotes apoptosis in embryonic hippocampal neurons. *J Neurosci.* 32, 2790-803.
- Sekiyama, Y., et al., 2012. Functional gene expression analysis of tissue-specific isoforms of Mef2c. *Cell Mol Neurobiol.* 32, 129-39.
- Sendtner, M., et al., 1992. Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature.* 360, 757-9.
- Shalizi, A., et al., 2006. A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. *Science.* 311, 1012-7.
- Swanson, B. J., et al., 1998. Characterization of myocyte enhancer factor 2 (MEF2) expression in B and T cells: MEF2C is a B cell-restricted transcription factor in lymphocytes. *Mol Immunol.* 35, 445-58.
- Tremolizzo, L., et al., 2013. Exploring limits of neuropsychological screening in ALS: the FAB problem. *Amyotroph Lateral Scler Frontotemporal Degener.* 14, 157-8.
- Tremolizzo, L., et al., 2014. Whole-blood global DNA methylation is increased in amyotrophic lateral sclerosis independently of age of onset. *Amyotroph Lateral Scler Frontotemporal Degener.* 15, 98-105.
- Volkenning, K., et al., 2009. Tar DNA binding protein of 43 kDa (TDP-43), 14-3-3 proteins and copper/zinc superoxide dismutase (SOD1) interact to modulate NFL mRNA stability. Implications for altered RNA processing in amyotrophic lateral sclerosis (ALS). *Brain Res.* 1305, 168-82.
- Wang, Y., et al., 2007. Brain-derived neurotrophic factor stimulates the transcriptional and neuroprotective activity of myocyte-enhancer factor 2C through an ERK1/2-RSK2 signaling cascade. *J Neurochem.* 102, 957-66.

- Wilker, P. R., et al., 2008. Transcription factor Mef2c is required for B cell proliferation and survival after antigen receptor stimulation. *Nat Immunol.* 9, 603-12.
- Williams, A. H., et al., 2009. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science.* 326, 1549-54.
- Yu, Y. T., et al., 1992. Human myocyte-specific enhancer factor 2 comprises a group of tissue-restricted MADS box transcription factors. *Genes Dev.* 6, 1783-98.
- Zhao, W., et al., 2013. Immune-mediated mechanisms in the pathopropagation of amyotrophic lateral sclerosis. *J Neuroimmune Pharmacol.* 8, 888-99.
- Zhao, W., et al., 2015. TDP-43 activates microglia through NF- κ B and NLRP3 inflammasome. *Exp Neurol.* 273, 24-35.

Chapter 4 - Conclusions

RNA metabolism is a multistep process including several different areas well known to be involved in ALS pathogenesis (Strong, 2010). In this study we have investigated two particular aspects potentially linked with transcriptional alterations occurring in ALS. First, we analysed the potential role of microRNAs as define pathological hallmark conserved in different disease models. With this aim, we have not only assessed a panel of miRNAs to identify any possible alteration in their expression between patients and controls, but we have identified a common set of two microRNAs altered in a common manner in different ALS models. Our findings showed that miR-129-5p and miR-200c were upregulated in both neuroblastoma cell lines and in transgenic mice expressing the pathological protein SOD1(G93A). Moreover, these microRNAs were also increased in PBMCs obtained from sporadic ALS patients with respect to healthy controls. Consequently, miR-129-5p and miR-200c could be considered as the “*lowest common multiple*” of alterations afflicting miRNAs, indicating probably a nodal event in ALS pathogenesis, since their expression were altered in both cellular and animal familial ALS models and in human samples from sporadic patients. Although further investigations will be performed to confirm if transcriptional alterations are selective for specific miRNAs or the final event of a global dysregulation, the altered expression of miR-129-5p and miR-200c previously reported could have direct consequences on several molecular targets. Our results identified HUD like a direct target of miR-129-5p and a lack of its regulation was able to cause alterations in neurogenesis, nerve development, and motor and sensory functions. Alterations affecting miR-129-5p function could directly alter neuronal morphology and function, offering a possible explanation of the

involvement of miR-129-5p in the pathogenesis of disease. Moreover, further evidences could be raised from the identification of other putative targets of miR-129-5p elucidating any more mechanisms clarifying the role of this miRNA in ALS.

The second part of the study was dedicated to elicitate a possible involvement of two transcriptional factors playing important roles in muscle and neuronal differentiation in the ALS context. Our findings reported evidences supporting another type of transcriptional alterations occurring in ALS. Indeed, we showed a systemic alterations of MEF2D and MEF2C pathways tipical of both sporadic patients and subjects with mutations in SOD1 gene. The expression levels of MEF2 downstream targets were found altered in patients and, although their exact function in peripheral cells is not clearly understood, they could cause any lack of functions in immune cells with possible consequences leading to a pathological condition. Our results showed increased MEF2D and MEF2C mRNA levels, altered subcellular localization and a reduction of their transcriptional activity. We have hyphotesized that HDACs proteins, which are well known to be up-regulated in SOD1(G93A) mice and in sporadic ALS patients (Bruneteau et al., 2013; Cohen et al., 2009; Cohen et al., 2007), could be responsible for MEF2 alterations (Figure 1).

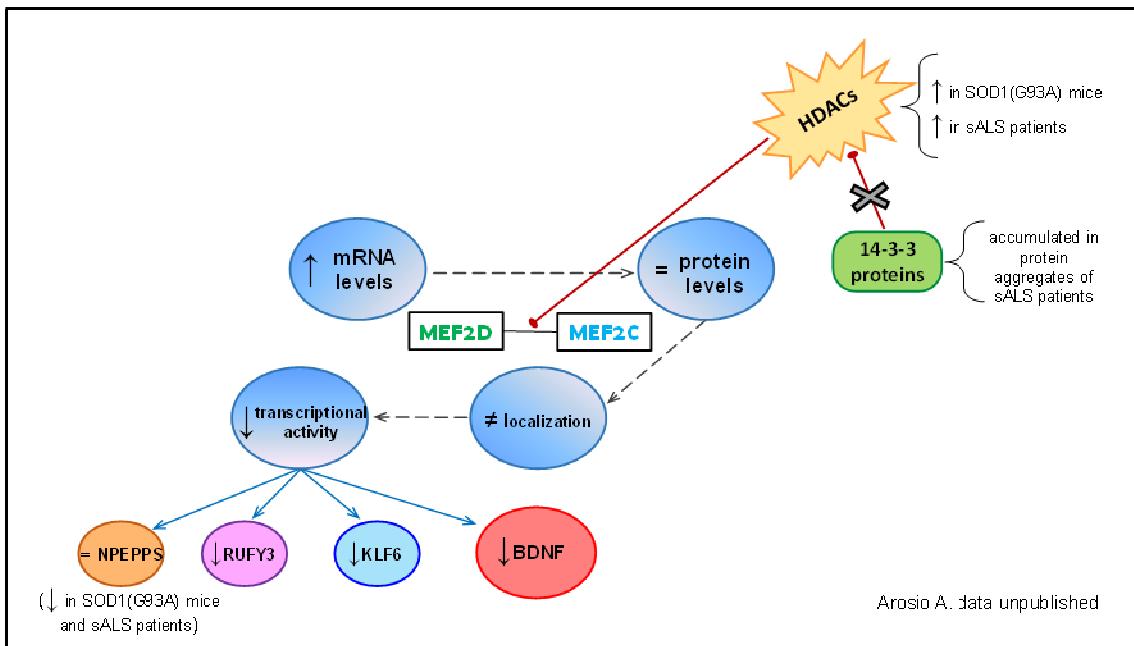


Figure 1

Schematic illustration representing alterations affecting MEF2D and MEF2C pathways previously reported in PBMCs obtained from sporadic and SOD1+ ALS patients. (Arosio et al., data unpublished)

Furthermore, 14-3-3- proteins, which are able to sequestre HDACs disrupting their repression on MEF2 proteins, were found to be accumulated in protein aggregates tipically observed in sporadic ALS patients (Volkening et al., 2009). Thus, 14-3-3 proteins are sequestred and can not prevent the inhibitory action of HDACs. Nevertheless, the inhibitory action of HDACs is not the only molecular mechanism potentially involved in MEF2 alterations. In fact, microRNAs could be responsible of unaltered protein levels showed despite the strong increase of MEF2D and MEF2C mRNA levels.

In this study we reported two altered mechanisms concernig RNA metabolism. Although these two exemples of transcriptional alterations are very different and charactherized by dissimilar mechanisms of actions, a possible link between microRNAs and MEF2 alterations could exists. Indeed, preliminary bioinformatics analysis showed that the 3'UTR of MEF2D gene could be potentially recognized by several miRNAs and amoung these there are miR-129-5p and miR-200c, which we have previously identified increased in sALS patients and in SOD1(G93A) *in vitro* and *in vivo* models. Indeed, a conserved binding site for miR-129-5p and miR-200c was identified in 3'UTR region of MEF2D (Figure 2), suggesting a possible regulatory mechanism involving all these parameters.

Figure 2

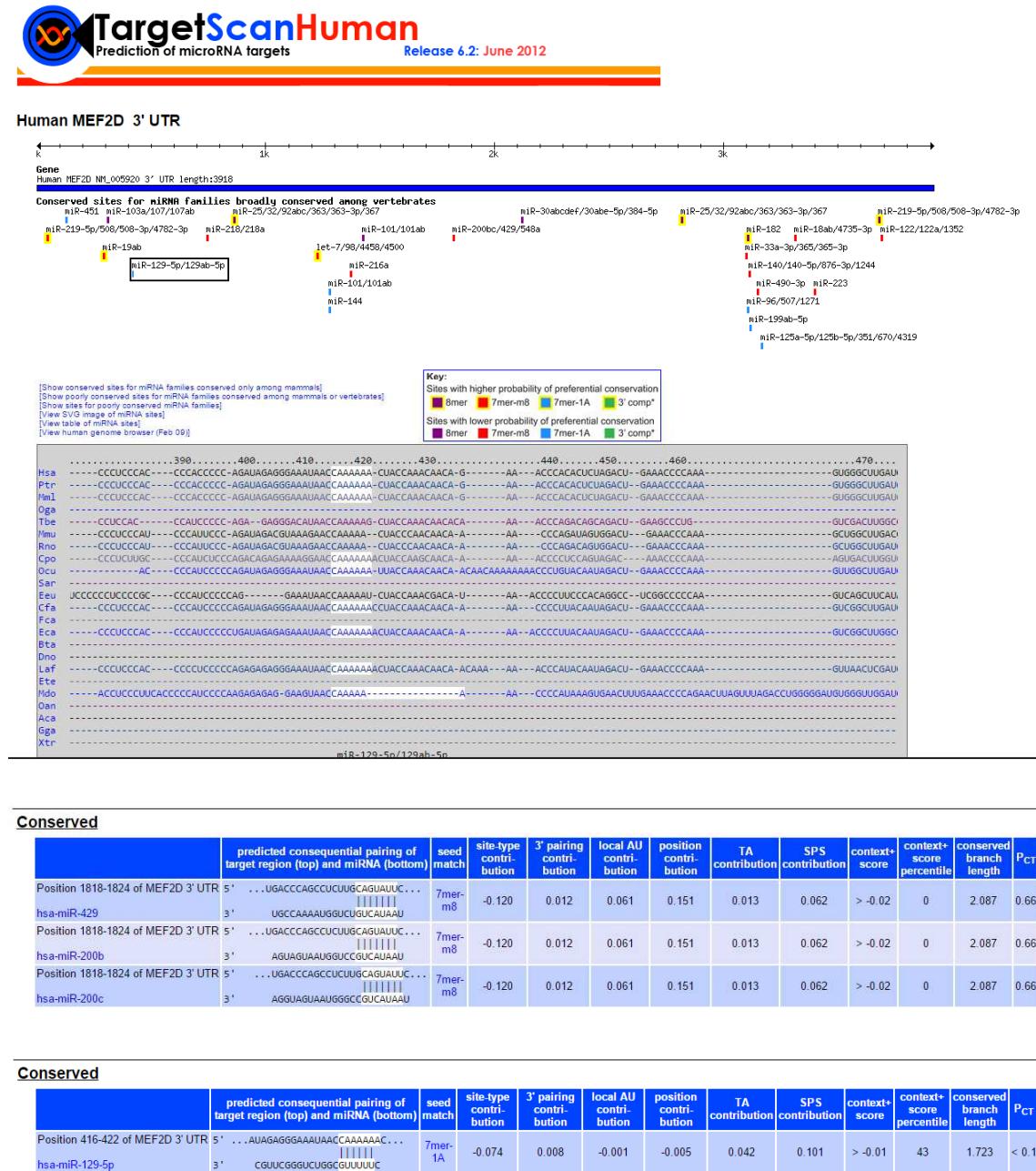


Figure 2

Schematic illustration showing several miRNAs potentially able to recognize the 3'UTR region of MEF2D gene (above). Figures reporting the binding site in MEF2D gene for miR129-5p and miR200c (below).

Evidences supporting the miRNA-mediated regulation of MEF2 proteins are largely increased in the last few years. Among these, it is possible to identify a group of miRNAs already demonstrated to be altered in ALS and potentially able to regulate MEF2 proteins or other molecules implicated in its regulation. Many of these miRNAs could potentially play an important role in the pathogenesis of disease, directly repressing MEF2D or MEF2C causing the reduction in their transcriptional activity previously described in the *Chapter 3* (Figure 3). For example, miR-155 and miR-338-3p were found increased in circulating cells of sporadic ALS patients (Butovsky et al., 2012; De Felice et al., 2012) and they were identified as potential regulators of MEF2 proteins using a bioinformatic prediction tool (Target Scan Human, version 7.0 released in August 2015). Moreover, miR-155 was already reported to inhibit MEF2 activity during muscle differentiation (Seok et al., 2011). Since miR-155 was previously reported to be increased in the spinal cord and in monocytes of sporadic ALS patients and in transgenic SOD1(G93A) mice where its repression with anti-miRs significantly prolonged survival (Butovsky et al., 2012; Koval et al., 2013), it could be hypothesized that miR-155 negatively influences disease progression. Furthermore, miR-155 was identified to negatively regulate BDNF (Vareni et al., 2014) which is known to favor motor neuron survival.

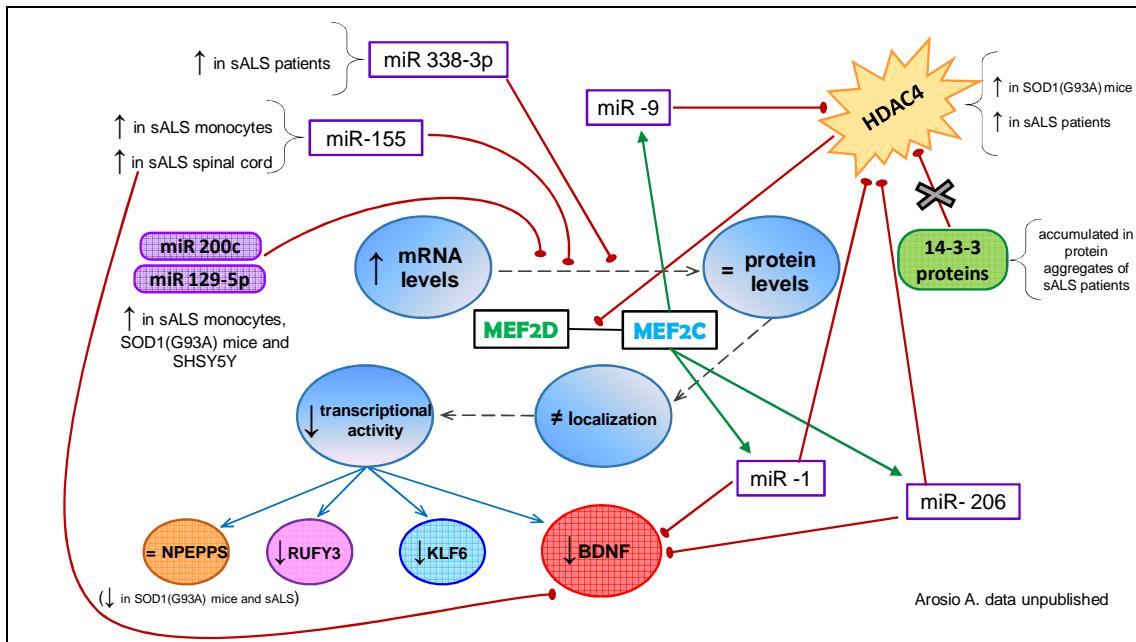
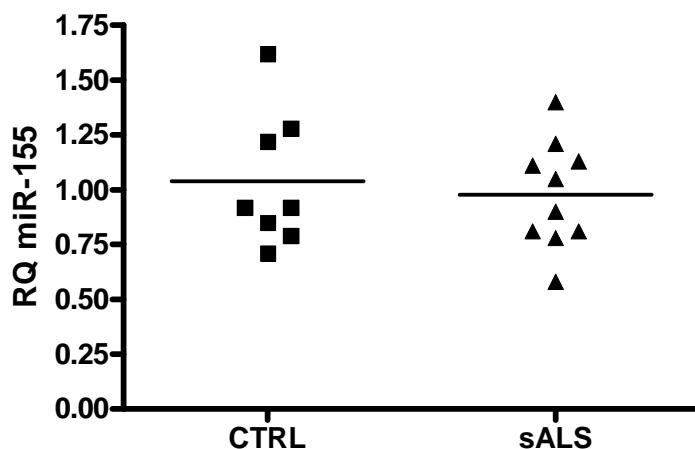


Figure 3

Schematic illustration representing a possible mechanism of regulation of MEF2 proteins mediated by microRNAs known to be altered in ALS. (Arosio et al., data unpublished)

In conclusion, miR-155 and miR-338-3p potentially regulate MEF2 transcriptional activity and it could be hypothesized that they negatively affect disease progression in a MEF2-mediated manner, for example directly targeting BDNF or reducing its transcription via MEF2D and MEF2C inhibition; but to confirm it further investigations will be necessary. To confirm that miR-155 was upregulated in PBMCs of ALS patients employed in our study and showing increased MEF2 levels, we assessed miR-155 expression in a small cohort of 10 sALS and 8 healthy controls. These preliminary data failed to show the increased miR-155 levels hypothesized (Figure 4) probably due to the too small cohort of subjects recruited; for this reason further analysis employing a bigger group of subjects will need to strengthen the analysis.

Figure 4



No differences in mir-155 expression levels were shown in PBMCs of sporadic ALS patients and healthy controls (CTRL n=8; sALS n=10) (Arosio et al., data unpublished).

On the other hands, MEF2 proteins also positively regulate some microRNAs able to inhibit HDAC proteins in a positive feed back loop. MEF2C was reported to induce during muscle differentiation the expression of miR-1 and miR-206 (Gagan et al., 2012), two microRNAs already investigated in ALS and previously identified to be able to negatively regulate HDAC4 proteins (Dai et al., 2015; King et al., 2014; Williams et al., 2009; Winbanks et al., 2013; Winbanks et al., 2011). HDAC4 sequestration by microRNAs prevents the inhibitory effect on MEF2 transcriptional activity sustaining this positive autoregulatory loop. Similarly, MEF2C also promotes miR-9 induction which inhibits HDAC4 (Davila et al., 2014), reinforcing the MEF2-miRNAs-HDACs axis. Moreover, miR-206 regulates BDNF (Lee et al., 2012; Miura et al., 2012; Tian et al., 2014; Yang et al., 2014) suggesting a further mechanism concerning the regulation of this neurotrophin directly or via MEF2 proteins. Both miR-206 and miR-9 were well known to be altered in both SOD1(G93A) mice and in ALS patients (Bruneteau et al., 2013; Marcuzzo et al., 2015; Russell et al., 2013; Toivonen et al., 2014; Williams et al., 2009;

Zhang et al., 2013). Taken together, all these evidences suggest that this complex cascade of molecular mechanisms involving MEF2 proteins, microRNAs and HDACs could represent a potential target of investigation to elucidate the real impact of this pathway in ALS and to clarify if it could become object of a possible pharmacological modulation.

References

- Bruneteau, G., et al., 2013. Muscle histone deacetylase 4 upregulation in amyotrophic lateral sclerosis: potential role in reinnervation ability and disease progression. *Brain.* 136, 2359-68.
- Butovsky, O., et al., 2012. Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. *J Clin Invest.* 122, 3063-87.
- Cohen, T. J., et al., 2009. The deacetylase HDAC4 controls myocyte enhancing factor-2-dependent structural gene expression in response to neural activity. *FASEB J.* 23, 99-106.
- Cohen, T. J., et al., 2007. The histone deacetylase HDAC4 connects neural activity to muscle transcriptional reprogramming. *J Biol Chem.* 282, 33752-9.
- Dai, Y., et al., 2015. The role of microRNA-1 and microRNA-206 in the proliferation and differentiation of bovine skeletal muscle satellite cells. In *Vitro Cell Dev Biol Anim.*
- Davila, J. L., et al., 2014. A positive feedback mechanism that regulates expression of miR-9 during neurogenesis. *PLoS One.* 9, e94348.
- De Felice, B., et al., 2012. A miRNA signature in leukocytes from sporadic amyotrophic lateral sclerosis. *Gene.* 508, 35-40.
- Gagan, J., et al., 2012. Notch3 and Mef2c proteins are mutually antagonistic via Mkp1 protein and miR-1/206 microRNAs in differentiating myoblasts. *J Biol Chem.* 287, 40360-70.
- King, I. N., et al., 2014. The RNA-binding protein TDP-43 selectively disrupts microRNA-1/206 incorporation into the RNA-induced silencing complex. *J Biol Chem.* 289, 14263-71.
- Koval, E. D., et al., 2013. Method for widespread microRNA-155 inhibition prolongs survival in ALS-model mice. *Hum Mol Genet.* 22, 4127-35.
- Lee, S. T., et al., 2012. miR-206 regulates brain-derived neurotrophic factor in Alzheimer disease model. *Ann Neurol.* 72, 269-77.
- Marcuzzo, S., et al., 2015. Up-regulation of neural and cell cycle-related microRNAs in brain of amyotrophic lateral sclerosis mice at late disease stage. *Mol Brain.* 8, 5.
- Miura, P., et al., 2012. Brain-derived neurotrophic factor expression is repressed during myogenic differentiation by miR-206. *J Neurochem.* 120, 230-8.
- Russell, A. P., et al., 2013. Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis. *Neurobiol Dis.* 49, 107-17.
- Seok, H. Y., et al., 2011. miR-155 inhibits expression of the MEF2A protein to repress skeletal muscle differentiation. *J Biol Chem.* 286, 35339-46.
- Strong, M. J., 2010. The evidence for altered RNA metabolism in amyotrophic lateral sclerosis (ALS). *J Neurol Sci.* 288, 1-12.
- Tian, N., et al., 2014. MiR-206 decreases brain-derived neurotrophic factor levels in a transgenic mouse model of Alzheimer's disease. *Neurosci Bull.* 30, 191-7.
- Toivonen, J. M., et al., 2014. MicroRNA-206: a potential circulating biomarker candidate for amyotrophic lateral sclerosis. *PLoS One.* 9, e89065.
- Varendi, K., et al., 2014. miR-1, miR-10b, miR-155, and miR-191 are novel regulators of BDNF. *Cell Mol Life Sci.* 71, 4443-56.
- Volkenning, K., et al., 2009. Tar DNA binding protein of 43 kDa (TDP-43), 14-3-3 proteins and copper/zinc superoxide dismutase (SOD1) interact to modulate NFL mRNA stability. Implications for altered RNA processing in amyotrophic lateral sclerosis (ALS). *Brain Res.* 1305, 168-82.
- Williams, A. H., et al., 2009. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science.* 326, 1549-54.
- Winbanks, C. E., et al., 2013. miR-206 represses hypertrophy of myogenic cells but not muscle fibers via inhibition of HDAC4. *PLoS One.* 8, e73589.
- Winbanks, C. E., et al., 2011. TGF-beta regulates miR-206 and miR-29 to control myogenic differentiation through regulation of HDAC4. *J Biol Chem.* 286, 13805-14.

Yang, X., et al., 2014. MicroRNA expression profile and functional analysis reveal that miR-206 is a critical novel gene for the expression of BDNF induced by ketamine. *Neuromolecular Med.* 16, 594-605.

Zhang, Z., et al., 2013. Downregulation of microRNA-9 in iPSC-derived neurons of FTD/ALS patients with TDP-43 mutations. *PLoS One.* 8, e76055.

Chapter 5 -

Additional Results & Further Perspectives

In this study we have analyzed the role of transcriptional alterations in ALS investigating a common set of microRNAs dysregulated in different ALS models and exploring the possible involvement of MEF2 proteins in the pathogenesis of disease. Alterations in RNA metabolism are crucial moment in ALS pathogenesis but are not the only phenomena potentially involved in the disease onset. Indeed, while we were carrying out this study we decided to develop two collateral and cognate topics, which will be described below. First, we previously reported decreased levels of BDNF in PBMCs probably due to a reduced MEF2 transcriptional activity (Arosio et al., data unpublished) and we discussed how many miRNAs altered in ALS can regulated BDNF expression. For this reason, a dedicated section to explore the role of BDNF in ALS will be discuss below, while the second part of this chapter is concern to the role of autophagy in ALS. Indeed, MEF2D is a specific substrate of the chaperone mediated autophagy (CMA) a degradative pathway which also contributes to the degradation of TDP-43 and never investigated in ALS; thus to explore the activity of this catabolic process in our samples we have performed dedicated analysis.

5.1 BDNF and hormones in ALS

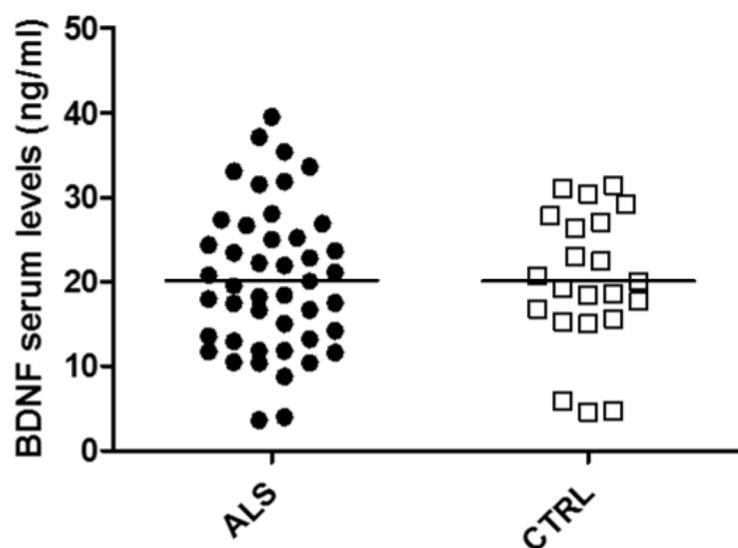
Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, a group of cytokines involved in several aspects of neuronal and extra-neuronal life. BDNF is synthesized as precursor proteins, pro-BDNF, packed into secretory vesicles and released both constitutively and upon stimulation. Cleavage by proteases, such as furin or proconvertases, produces mature neurotrophins. BDNF exerts its functions mainly at the synaptic cleft, through interaction with two types of receptor: the tropomyosin related kinase B (TrkB) receptor and the p75 neurotrophin receptor (p75NTR). In adult brains, mature BDNF predominantly interacts with TrkB inducing neuronal survival and synaptic plasticity. On the other hand, pro-BDNF binds with higher affinity to p75NTR, a member of the tumor necrosis factor superfamily of receptors, prompting apoptosis and a shrinkage of dendritic spines. Pro-BDNF and BDNF metabolism and functions outside the nervous system are less well characterized. However it was shown that BDNF passes the blood-brain barrier (BBB) bidirectionally (Pan et al., 1998; Sartorius et al., 2009) so that it may “leak” outside the central nervous system (CNS), reach the blood stream and affect other tissues expressing TrkB receptor. Vice-versa, peripherally produced BDNF may affect availability of such neurotrophin within the CNS. Plasma BDNF is rapidly degraded by proteases, or uptaken by platelets which in turn release it upon stimulation, so that serum levels of BDNF are 100-fold those of plasma. Interestingly, alterations of neurotrophin availability has been advocated as a pathogenic mechanism underlying ALS neurodegeneration: BDNF, in particular has been shown to impact survival of motor neurons by both in vitro and in vivo models of neuron injury or death (Henderson et al., 1993; Ikeda et al., 1995; Koliatsos et al., 1993;

Mitsumoto et al., 1994; Novikova et al., 1997; Sendtner et al., 1992). However, therapeutic trials of BDNF infusion, failed to show significant clinical benefit (1999; Ochs et al., 2000). Immunohistochemical post-mortem studies of motor cortex and spinal cord samples of ALS patients showed that the remaining mass of motor neurons retains the ability to express BDNF and its receptors at levels comparable to those of controls, however a switch in their susceptibility from BDNF and NT-3 to NGF may have occurred (Duberley et al., 1997; Kawamoto et al., 1998; Nishio et al., 1998). Similar studies performed on muscle biopsies and cerebrospinal fluid (CSF) held mixed results (Grundström et al., 1999; Grundström et al., 2000; Küst et al., 2002), one of them, however, identified a pattern of BDNF/NGF expression, with longer disease duration being characterized by a decreased BDNF and increased NGF expression within muscle cells. To our knowledge only two studies measured peripherally circulating BDNF and only one of them in serum (Angelucci et al., 2004; Ilzecka and Stelmasiak, 2002). This study, specifically, failed to show significant differences between ALS patients and a group of lumbar disc disease controls. On the other hand, circulating levels of BDNF were found altered in several neurological and psychiatric disorders (Teixeira et al., 2010). Lower BDNF serum levels were shown, for example, in FTLD patients versus healthy controls but not Alzheimer disease (AD) patients (Ventriglia et al., 2013). Albeit the circulating levels of this neurotrophin might not be different, overall, in ALS patients with respect to healthy controls, we may hypothesize that BDNF might characterize differentially expressed phenotypic traits. Therefore, we assessed BDNF serum levels in 45 ALS outpatients implementing a multidimensional characterization based on four, *a priori* chosen, disease-defining axes: ALS bulbar/spinal subtype, speed of disease progression, cognition and mood dysfunctions.

Considering the whole cohort of ALS patients, serum BDNF levels were similar, overall, with respect to controls (**Figure 1**). Nevertheless, disease staging (defined according to ALSFRS-R scores) correlated with BDNF serum levels ($r= 0.39$, $p<0.01$) and patients with ALSFRS-R scores below of the median value 32 ($n=21$ [46.6%]) had ~25% lower BDNF serum levels with respect to patients scoring above 32 ($p=0.01$) (**Figure 2A-2B**).

Figure 1

Serum BDNF levels in ALS patients and healthy controls (CTRL).

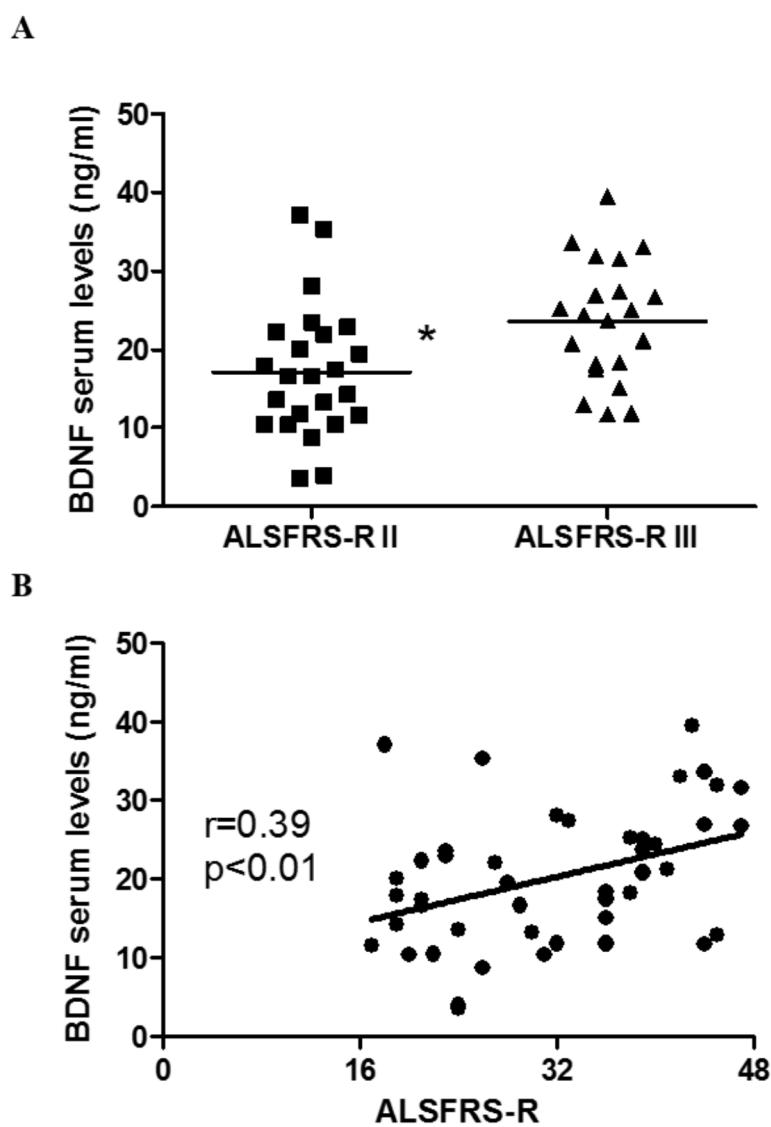


Tremolizzo et al., 2015; Neurodegenerative Diseases. *In press*

Lastly, we analyzed BDNF serum levels investigating any possible correlations with multidimensional correlates in ALS. Bulbar ($n=7$) versus spinal ($n=38$) onset patients did not significantly differ in terms of BDNF serum levels (22.7 ± 9.3 vs. 19.7 ± 8.6 ng/ml $p=0.42$, mean \pm SD, respectively). Analyzing cognitively impaired versus spared patients no differences were found as well as comparing fast versus slow progressors.

Figure 2

(A) serum BDNF is ~25% lower in ALS patients with ALSFRS-R scores belonging to the more impaired group (scores >16 and ≤ 32 , defined as II) with respect to those patients with ALSFRS-R scores (>32) belonging to the less impaired group (defined as III; note that no patients scored below 17), $p=0.01$ Student's t-test; (B) correlation between ALSFRS-R scores and serum BDNF levels in ALS patients.

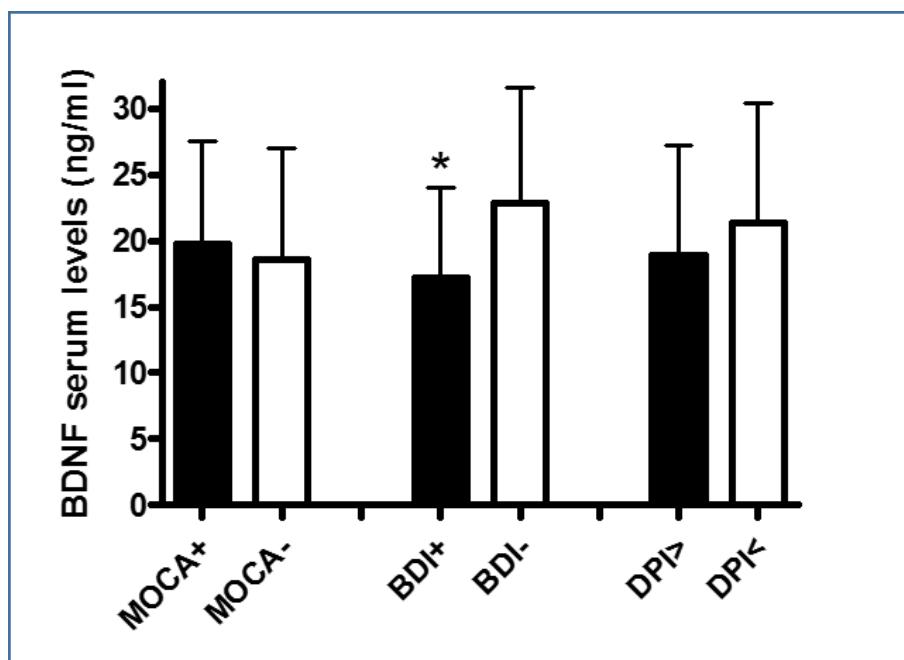


Tremolizzo et al., 2015; Neurodegenerative Diseases. *In press*

Accordingly, neurotrophin serum levels did not correlate with both the Montreal Cognitive Assessment score (MoCA) and with the Disease Progression Index (DPI). On the other hand, ALS patients showing a depressive trait with (n=18 [40%]) had ~25% lower BDNF serum levels with respect to non-depressed patients ($p<0.03$, see **Figure 3**) and correlated with the BDI scores ($r=-0.37$, $p<0.03$).

Figure 3

Serum BDNF levels in ALS patients stratified according to the pre-specified multidimensional parameters. Data are presented as mean \pm SD. Cut-offs defining altered scores were: Beck depression inventory >9 (BDI+); MoCA <26 (MoCA-). Fast progressors (DPI>) had a score >0.39. * $p<0.03$ two-tailed Student's t-test.

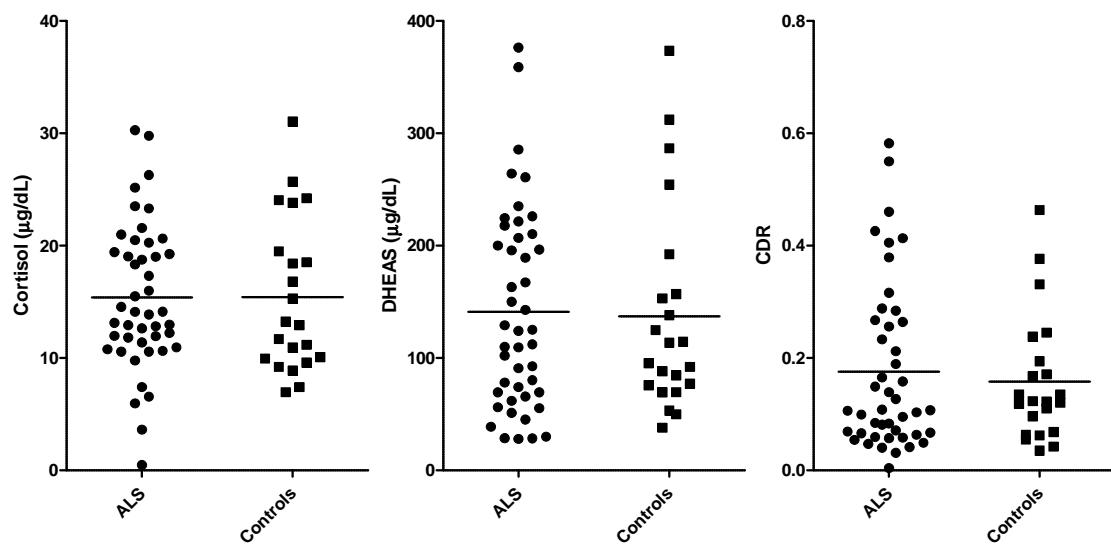


Tremolizzo et al., 2015; Neurodegenerative Diseases. *In press*

This study was designed in order to get meaningful insights on the determinants of phenotypic variability in ALS. In conclusion, we initially failed to report differences in serum BDNF between ALS patients and healthy controls, extending previous findings published on smaller series (Ilzecka and Stelmasiak, 2002). However, BDNF was downregulated in depressed patients. Accordingly, BDNF decrease has already been reported in depressed patients (de Azevedo Cardoso et al., 2014) and a recent meta-analysis showed that altered peripheral BDNF levels are associated to ongoing depressive disorders (Molendijk et al., 2014). Clearly this implies that mood dysfunction in ALS is not only a mere adaptive phenomenon to a progressive overwhelming disability, but a complex phenotypic trait regulated by specific biological mechanisms that might be amenable of early dedicated interventions. Interestingly, however, an important determinant of neurotrophin concentration is stress exposure: accordingly, converging lines of research show that an altered production during stressful periods of life could nourish the biological dysfunctions underlying depressive disorders (Kimpton, 2012). In line with this hypothesis, steroid hormones, such as cortisol and dehydroepiandrosterone (DHEA), involved in mediating stress effects and playing key roles in the pathophysiology of mood disorders, are also known to modulate the production of BDNF (Pluchino et al., 2013). Thus, to investigate if the decreased BDNF expression described in serum of depressed ALS patients could be caused by alterations involving these two stress hormones, we assessed the expression levels of cortisol, DHEA and its sulfate metabolite (DHEAS) and the cortisol/DHEAS Ratio (CDR) in serum of our patients (**Figure 4**) but no significant differences emerged.

Figure 4

Serum cortisol, DHEAS and cortisol/DHEAS ratio (CDR) levels in ALS patients.



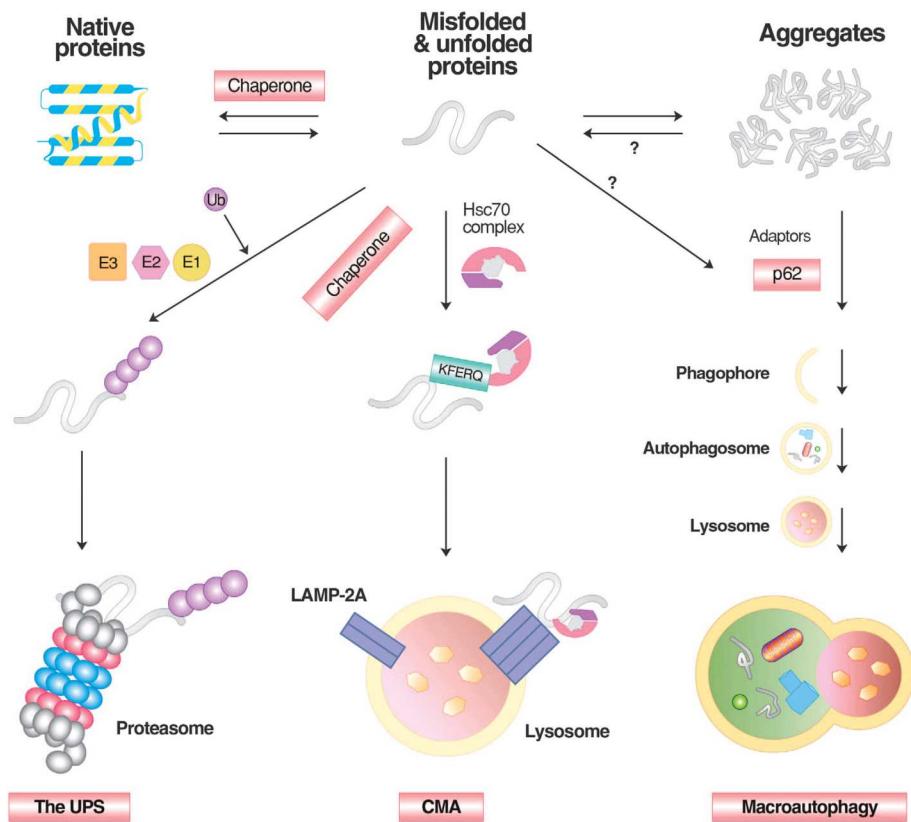
Furthermore, to better characterize the bioavailability of these hormones, the expression levels of specific hormone-binding proteins will be assessed. We will specifically evaluate the circulating levels of albumin, which binds DHEAS and transports it in the bloodstream. Moreover, we have also planned to assess levels of testosterone and its protein carrier Sex hormone-binding globulin (SHBG) in ALS patients with respect to controls.

In conclusion, we report here that BDNF dysfunction might lie beneath selected phenotypic traits in ALS, and in particular depressed mood and advanced functional loss (Tremolizzo et al., 2015; *In press*). Further studies will clarify whether these changes might become clinical tools suitable for guiding specific therapeutic interventions.

5.2 Chaperone mediated autophagy (CMA) in ALS

Misfolded proteins generated in various cellular compartments, including the cytoplasm, nucleus and endoplasmic reticulum (ER), are efficiently removed by quality control systems composed of the ubiquitin (Ub)-proteasome system (UPS), chaperone mediated autophagy (CMA) and macroautophagy (**Figure 5**). The first line of defense in degrading soluble misfolded proteins is the UPS, a selective proteolytic system in which substrates are tagged with Ub, unfolded into nascent polypeptide chains, and cleaved into short peptides while passing through the narrow chamber of the proteasome. Specific misfolded proteins that expose the KFERQ degradation signal can be degraded by the CMA, a branch of the autophagy-lysosome system (hereafter autophagy), in which substrates are selectively recognized by the chaperone heat-shock cognate 70 (hsc70) and directly delivered into lysosomes, leading to degradation by lysosomal hydrolases into aminoacids. Some misfolded proteins that escape the surveillance of the UPS and CMA or tend to form aggregates are directed to macroautophagy, a bulk degradation system in which substrates are segregated into autophagosomes which, in turn, are fused with lysosomes for degradation into aminoacids.

Figure 5



The degradation of misfolded proteins by various cellular proteolytic pathways. Misfolded proteins are initially recognized by molecular chaperones that deliver the substrates to the UPS, CMA or macroautophagy depending on the nature of misfolding, size and solubility. In general, soluble and monomeric misfolded proteins are primarily degraded by the UPS and CMA. In CMA, substrates carrying the KFERQ motif are recognized and bound by Hsc70 in association with chaperones. The substrates are subsequently delivered to the LAMP2 complex on the lysosomal membrane, translocated to the lumen, and degraded into amino acids by lysosomal hydrolases. Some of these misfolded proteins tend to form aggregates and are thus directed to macroautophagy. Misfolded protein substrates of macroautophagy are recognized by molecular chaperones such as Hsc70, ubiquitinated by Ub ligases, and delivered to the autophagic adaptor p62, leading to the formation of p62 protein bodies. The targeted protein aggregates associated with p62 are subsequently delivered to autophagic membranes for lysosomal degradation, when p62 interacts with LC3 on the autophagic membrane.

Among the three arms of autophagy, the targeted clearance of misfolded proteins is mainly mediated by CMA and macroautophagy. CMA is a selective proteolytic system in which specific misfolded proteins carrying the KFERQ motif are delivered to and degraded in lysosomes. This pentapeptide motif, found in ~ 30% of cytosolic proteins, is normally buried by protein folding, but it can be exposed on the surface by misfolding or partial unfolding. It is recognized by the chaperone Hsc70 associated with co-chaperones (Kiffin et al., 2004). The substrates are subsequently delivered to the CMA adaptor lysosomal membrane-associated protein 2A (lamp-2A) on the lysosomal membrane, unfolded, translocated into the lysosomal lumen and degraded into aminoacids. In degenerating neurons, CMA can be constitutively activated to compensate for impaired macroautophagy (Kaushik et al., 2008).

Concerning to ALS, although various pathogenic mechanisms have been proposed to sustain the pathogenesis of disease, a possible role for autophagy was observed in transgenic mouse models of fALS. For instance, in the transgenic SOD1(G93A) mouse, increased autophagy was observed during the presymptomatic stage (<90 days of age). Surprisingly, any significant changes in autophagy in the soma of motor neurons were not observed until the terminal stage in these mice (140 days of age)(Li et al., 2008). Although autophagy seems impaired in the ALS model, many researchers showed its induction in ALS mouse models (Morimoto et al., 2007; Song et al., 2012). In patients with sporadic ALS, the autophagy features were observed under electron microscopy in the cytoplasm of normal motor neurons and more frequently in degenerated motor neurons (Sasaki, 2011). Furthermore, small heat shock protein B8 (HSPB8) decreases the aggregation of mutant SOD1 and increases its solubility and clearance by enhancing

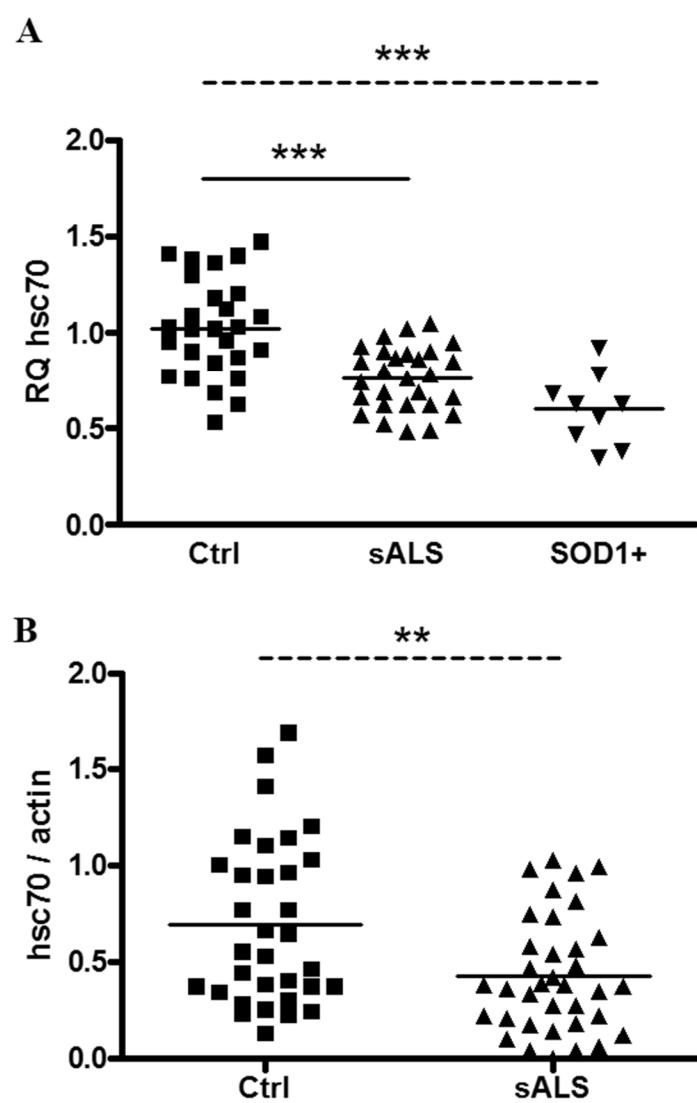
autophagy without affecting wild-type SOD1 turnover in the SOD1(G93A) ALS model mice (Crippa et al., 2010a; Crippa et al., 2010b). Other evidences sustaining an impairment of autophagy are related to the clearance of TDP-43, the main component of protein aggregates observed in sporadic ALS patients; TDP43 turnover is known to be enhanced by autophagy activation and that autophagy-activating compounds improve TDP43 clearance and enhance survival in neuronal ALS models (Barmada et al., 2014). TDP-43 is well known to be degraded by UPS and macroautophagy; however a recent study recently showed that it is also degraded by the chaperone-mediated autophagy mediated through an interaction between hsc70 (also known as HSPA8) and ubiquitylated TDP-43 (Huang et al., 2014). The authors also reported that TDP-43 is cleaved by caspases and produces fragments with lower molecular weight, moreover TDP-25 was identified as the principal substrate necessary for formation of the cytosolic TDP-43-positive aggregates observed in patient' cells. Furthermore, MEF2D is a selective substrate of CMA (Yang et al., 2009) and we previously reported in ALS patients increased mRNA levels for this protein without significant changes in protein levels (Arosio et al., data unpublished) compatible with a possible increase of the degradative activity of its catabolic pathway.

Currently, no data exist about the CMA activity in ALS patients or in animal models of disease; for this reason a dedicated study with the collaboration of Prof. Poletti's group and Dr. Cereda's lab started to assess the CMA activity in ALS. Firstly, we have assessed the two principal parameters of CMA in PBMCs obtained from 30 sALS, 9 SOD1+ patients and 30 healthy controls age and sex matched. Our results showed a significant reduction in hsc70 mRNA levels in both sporadic and SOD1+ ALS patients with respect

to healthy controls (**Figure 6A**). Moreover decreased hsc70 proteins were also observed in sporadic ALS patients versus controls (**Figure 6B**). The other parameter of CMA is lamp2A and, since its binding to the substrate protein represents the limiting step of CMA, lamp2A protein levels have been shown to directly correlate with CMA activity (Cuervo and Dice, 2000a; Cuervo and Dice, 2000b).

Figure 6

Hsc70 gene and protein expression in PBMCs of sporadic patients (sALS), ALS patients with mutations in SOD1 gene (SOD1+) and healthy controls. **(A)** Decreased hsc70 mRNA levels in sALS and SOD1+ patients with respect to healthy controls (CTRL). Relative quantification (RQ) of hsc70 mRNA is calculated as ratio to β -actin. One-way ANOVA, followed by Tukey's multiple comparison test ($***p<0.001$; CTRLs n=30; sALS n=30; SOD1+ n=9). **(B)** Reduced hsc70 protein levels were shown in sporadic ALS patients versus controls. Hsc70 protein levels are expressed as ratio between target and beta-actin optical density, Two-tailed unpaired Student's *t*-test ($**p<0.01$; CTRLs n=30; sALS n=30).



We assessed mRNA and protein levels of lamp2A but we failed to show any differences between patients and controls (**Figure 7A-7B**). However, a negative correlation between lamp2A protein levels and disease duration, expressed in months, emerged (**Figure 8A**). Indeed, stratifying patients based on disease duration and dividing them in two groups (sALS patients with a disease duration lower than the median value of 18 months and patients with a disease duration > 18 months) we showed reduced lamp2A protein levels in patients with a longer disease duration (**Figure 8B**).

Our results evidenced a significant reduction in hsc70 protein levels in PBMCs of ALS patients due to a decreased mRNA synthesis. Conversely, lamp2A protein, which is the parameter representing the rate limit step of CMA activity, was unaltered excluding an impairment of this catabolic pathway, at least in initial phases of disease. As a matter of fact, patients with a longer disease duration had reduced lamp2A protein levels without changes in mRNA levels (data not shown) suggesting that a possible lack of CMA function could arise in the last steps of disease. Therefore, our results evidenced different regulation mechanisms for hsc70 and lamp2A: the cytosolic carrier was found decreased in ALS patients because its transcription is reduced with respect to controls and disease duration no

Figure 7

Lamp2A gene (**A**) and protein (**B**) expression is unchanged in PBMCs of sporadic patients (sALS), ALS patients with mutations in SOD1 gene (SOD1+) and healthy controls. Relative quantification (RQ) of lamp2A mRNA is calculated as ratio to β -actin (CTRLs n=30; sALS n=30; SOD1+ n=9). Protein levels are expressed as ratio between lamp2A and beta-actin optical density (CTRLs n=30; sALS n=30).

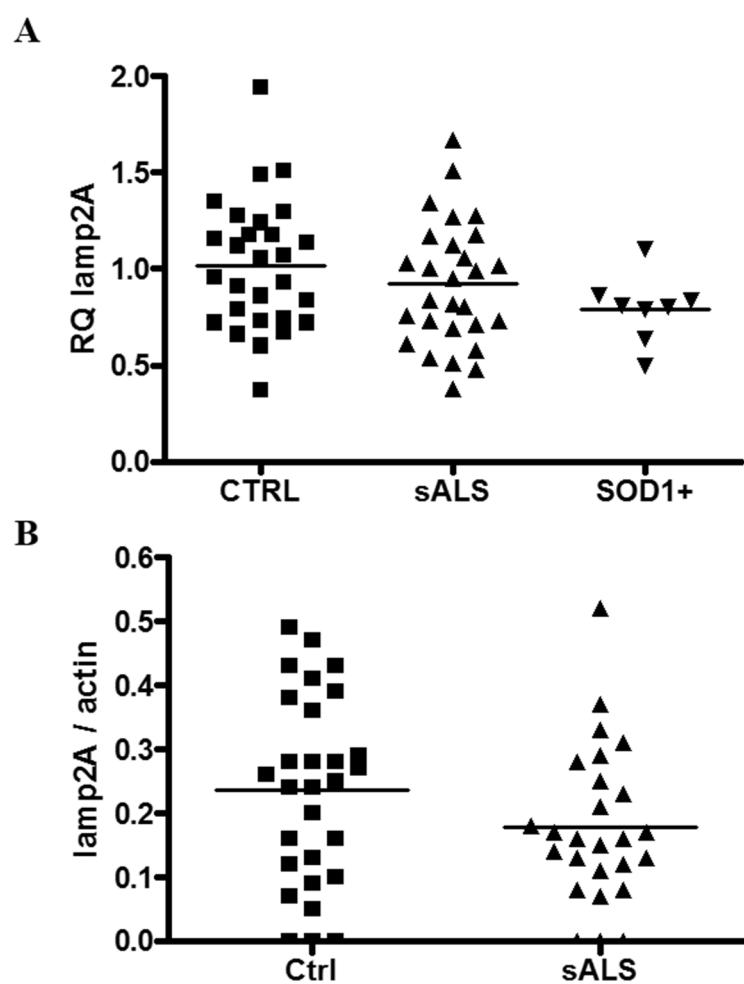
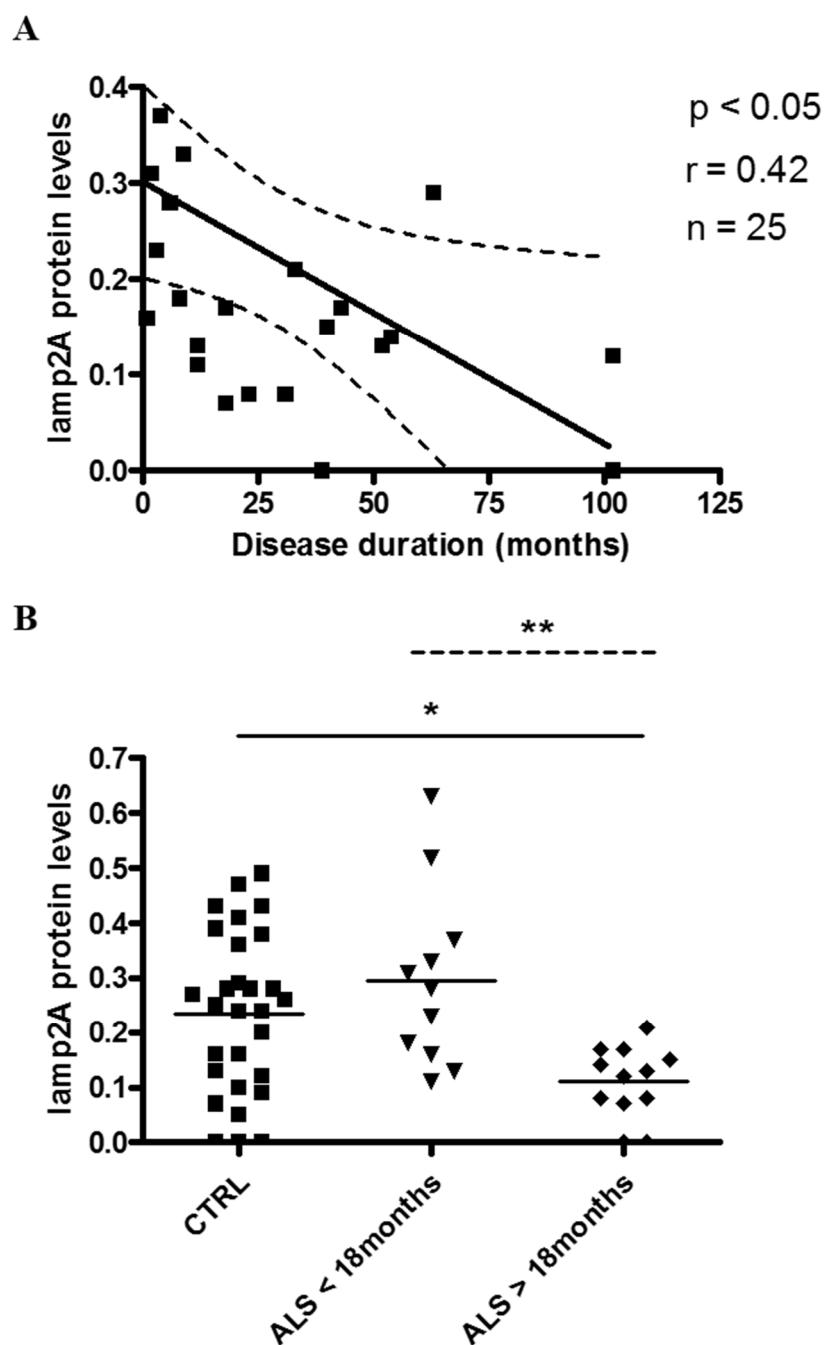


Figure 8

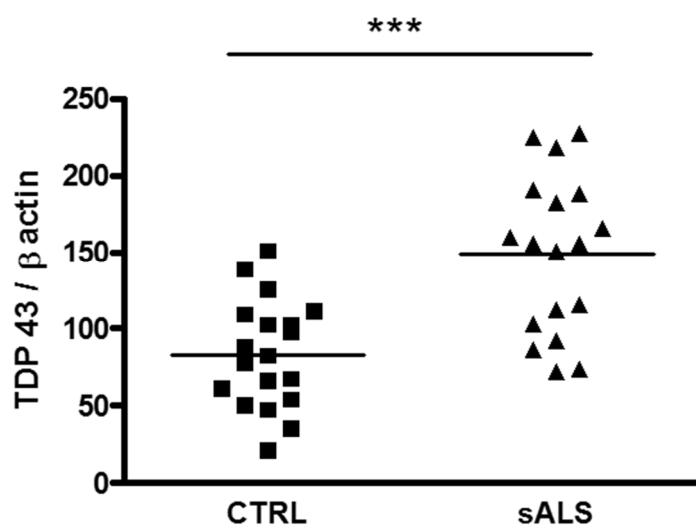
(A) Lamp2A protein levels negatively correlates with disease duration (months) ($r=0.42$, $p<0.05$, $n=25$). Moreover, although considering the whole cohort of ALS patients lamp2A protein levels were unchanged with respect to controls, stratifying patients based on the disease duration expressed in months (< of the median value of 18 months or > of 18 months), we showed decreased lamp2A protein levels in ALS patients with a long disease duration versus controls (B, $*p<0.05$; $**p<0.01$).



influences neither mRNA nor hsc70 protein levels (data not shown), while unaltered lamp2A protein levels suggests that CMA activity seems to be not altered in ALS patients until the last stages of disease where reduced lamp2A protein levels were evidenced in PBMCs of ALS patients. To assess in these cells the degradative activity of CMA we have investigated levels of its putative targets. Firstly, we evaluated TDP-43 protein levels and we showed a significant increment in PBMCs of sporadic ALS patients with respect to controls (**Figure 9**).

Figure 9

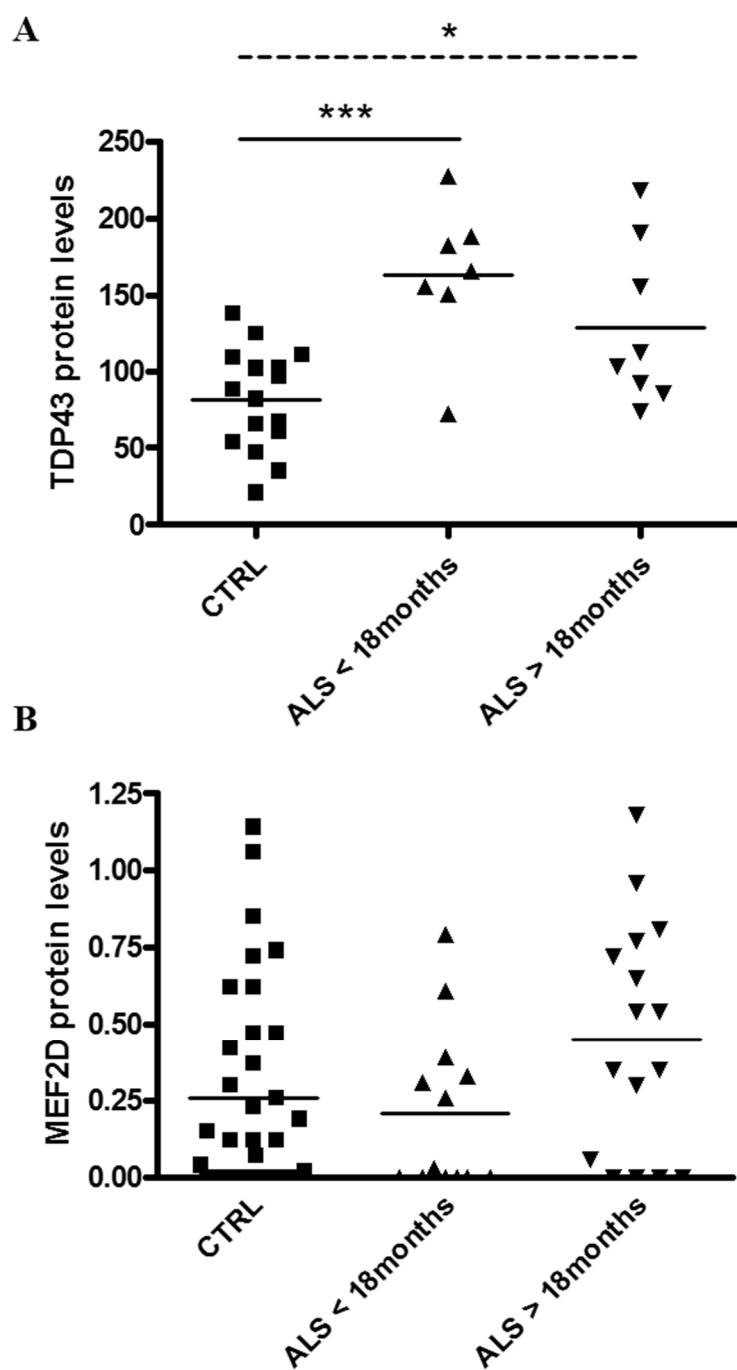
Increased TDP-43 protein levels in PBMCs of sALS patients with respect to controls. TDP-43 protein levels are expressed as ratio between target and beta-actin optical density, each value was calculated referred to the mean value of the controls loaded on each gel and expressed as %. (**p<0.001; CTRLs n=20; sALS n=20).



We hypothesize that increased TDP-43 protein levels reported in ALS PBMCs could be related to the protein aggregates TDP-43-positive described in the literature, suggesting that protein levels were increased because unfolded TDP-43 were accumulated in protein aggregates; but further investigations are needed to confirm this hypothesis. However TDP-43 clearance is carried out also by UPS and macroautophagy, so to better evaluate specifically CMA activity a specific target could be assessed. MEF2D is selectively degraded by CMA (Yang et al., 2009) but we previously described unaltered protein levels for this parameter between sALS and healthy controls (Arosio et al., data unpublished) suggesting that CMA seems to be not affected in ALS patients and TDP-43 protein levels were found increased probably due to an impairment of other degradative systems. Anyway, stratifying patients based on disease duration as previously described, we showed that TDP-43 protein levels were upregulated from the earliest stages of disease (**Figure 10A**) (in agreement with the literature describing TDP-43 aggregates like pathological hallmarks of ALS), while MEF2D showed no differences in protein levels between patients and controls even if MEF2D seems to be increased ($p=0.07$) in ALS patients with longer disease duration (**Figure 10B**), similarly to lamp2A.

Figure 10

CMA substrates stratified based on disease duration. (A) TDP-43 protein levels were increased in both patients with disease duration <18 months and in patients with a longer disease duration (*p<0.05; ***p<0.001). (B) MEF2D protein levels were unaltered between sALS and controls, but patients with a longer disease duration seems to have higher MEF2D levels even if without statistical significance (p=0.07).



Taken together, these results seem suggest that CMA is not affected in PBMCs of ALS patients in the earliest stages of disease, even if a possible impairment of the degradative activity of this pathway could arise during disease progression (Arosio et al., data unpublished). However, the hsc70 downregulation here described could affect CMA degradative efficiency reducing the amount of cytosolic carrier available to direct the substrate to the lysosome where it will be degraded. Furthermore, hsc70 is well known to play several function in the cell cooperating with various co-chaperone molecules; indeed, hsc70 is not only described in association with CMA but it is also involved in UPS system and macroautophagy. Hsc70 is involved in targeting proteins to ubiquitin/proteasome machinery interacting with two co-chaperones: BAG1 and CHIP (carboxyl-terminus of HSC70 interacting protein). Interaction between BAG1 and hsc70 leads to the activation of CHIP which acts as a chaperone associated ubiquitin ligase regulating protein quality control (Zhang et al., 2005). CHIP mediates the attachment of an ubiquitin chain to chaperone-bound client proteins and stimulates substrate degradation by proteasome. Moreover in the last years, evidence has also been acquired supporting a participation of hsc70 in autophagic pathways such as microautophagy. Recently, a microautophagy-like process involving hsc70, called endosomal microautophagy, has been described (Sahu et al., 2011). As in CMA, hsc70 binds to KFERQ motif-containing proteins, but instead of binding to a lysosomal membrane receptor, the chaperone directly associates through electrostatic interaction with the phosphatidylserine moieties present in the lipid bi-layer. Consequently, the protein substrate can be easily internalized and degraded through endosomal microautophagy. Finally, recent studies have highlighted an involvement of hsc70 in macroautophagy. During this process, large portions of cytosol are sequestered inside double-membrane

vesicles known as autophagosomes; the captured components are then degraded upon fusion of these structures with lysosomes. This catabolic pathway is relevant for the removal of different cytoplasmic components such as damaged organelles or intracellular protein aggregates (Wong et al., 2011). Hsc70, widely associated with these aggregates, targets them to the lysosomes through a particular macroautophagic process known as chaperone-assisted selective autophagy (CASA). The co-chaperone BAG3 binds hsc70, inducing ubiquitination of the protein substrate. Instead of being targeted for proteasomal degradation as described above, the complex is recognized by the macroautophagy receptor SQSTM1/p62 (sequestosome 1) protein. The subsequent binding of SQSTM1 to the autophagosomal membrane-anchored LC3, delivers this CASA substrate to the macroautophagy machinery for degradation (Arndt et al., 2010; Ulbricht et al., 2013). All these degradative pathways could be involved in ALS and eventual hsc70 alterations could directly affects the pathogenic mechanisms occurring during disease. As a matter of fact, hsc70 and its co-chaperone CHIP facilitates degradation of the ALS-linked mutant SOD1 in an ubiquitination-dependent manner (Urushitani et al., 2004). Studies with transgenic mouse models of ALS also suggested that the inclusions may be sequestered into ubiquitin-proteasome pathway and hsc70 may be involved in formation and/or degradation of these inclusions (Choi et al., 2004; Song et al., 2013; Wang et al., 2009; Watanabe et al., 2001; Zetterström et al., 2011). Anyway, hsc70 was also described to mediate the degradation of mutant SOD1 via autophagy. Indeed, the multiheteromeric complex HspB8/Bag3/Hsc70/CHIP interacts with mutant SOD1 and activates its autophagic removal (Crippa et al., 2010b). This process is strongly regulated by BAG3 which enhances autophagy activity, as indicated by the increased LC3 and p62 mRNA levels observed in skeletal muscles of transgenic SOD1(G93A) mice at symptomatic

stage (Crippa et al., 2013). In conclusion, hsc70 could determinate the final fate of its substrate conveying it to a specific catabolic pathway. To better understand these mechanisms and to assess the real impact of hsc70 in ALS we started a collaboration with Prof. Poletti's group to study in deep this topic and our preliminary results are described below.

Firstly, we investigated the two key regulators of macroautophagy: LC3 and p62 to assess if the induction of this pathway previously described in animal ALS model also occurred in peripheral cells obtained from blood patients. We showed a significant reduction of LC3 and p62 mRNA levels in both sporadic and SOD1+ ALS patients with respect to controls (**Figure 11**) and a positive correlation between mRNA levels of these two parameters was also observed in patients and in controls (**Figure 12**). Then, we investigated mRNA levels of BAG1 and BAG3 the co-chaperones of hsc70 that directing substrates to be degraded by UPS or autophagy, and we showed reduced BAG1 mRNA levels in both sporadic and SOD1+ ALS patients with respect to controls, while no changes in BAG3 mRNA levels were observed in patients (**Figure 13**).

Taken together, these preliminary results seem suggest that the hsc70 down-regulation previously described could afflict several degradative pathways. Although further investigations will be need to finely assess the functional status of macroautophagy and UPS system in PBMCs of ALS patients, reduced levels of hsc70 seem impair degradative activity. Alterations in protein quality control system could play a crucial role in ALS and are responsible for the accumulation of misfolded proteins and aggregates formation which are pathological hallmarks of ALS.

Figure 11

Reduced gene expression of LC3 (**A**) and p62 (**B**) in sporadic and SOD1+ ALS patients with respect to controls. Relative quantification (RQ) of mRNA target is calculated as ratio to β -actin (*p<0.05; **p<0.01 ***p<0.001; CTRLs n=30; sALS n=30; SOD1+ n=9).

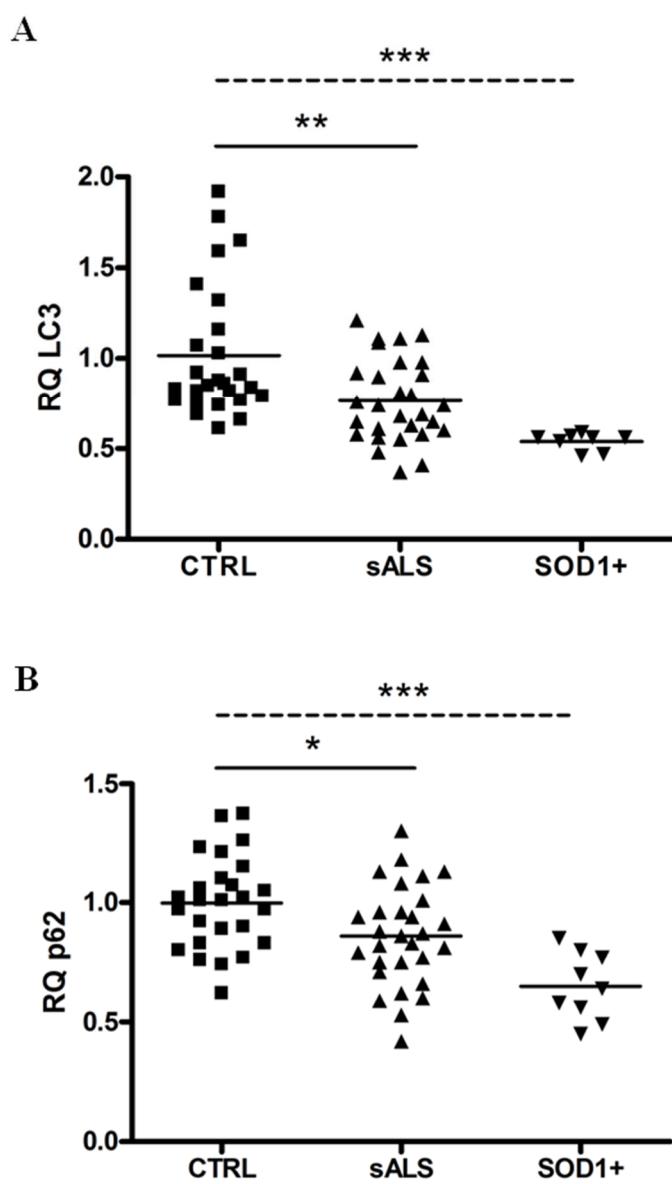


Figure 12

The two parameters of macroautophagy LC3 and p62 mRNA levels showed a positive correlation in PBMCs of healthy controls (**A**. $p<0.0001$; $r=0.77$; $n=30$) and sporadic ALS patients (**B**. $p<0.01$; $r=0.54$; $n=30$).

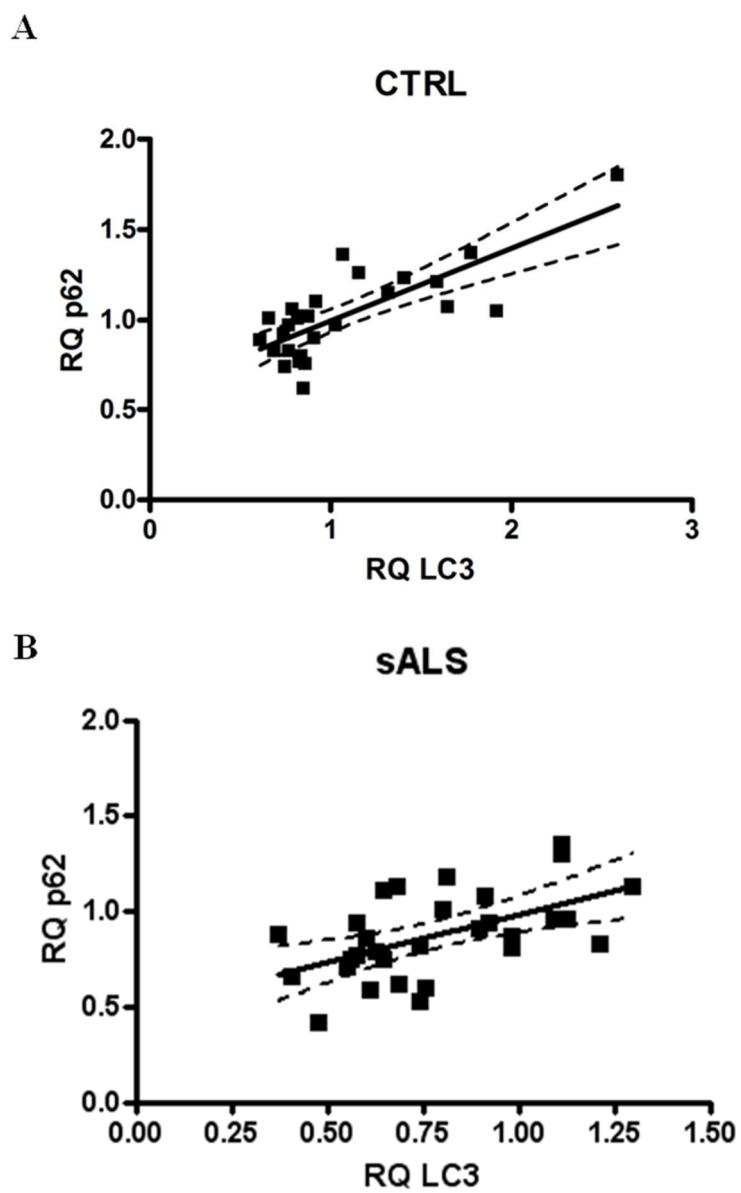
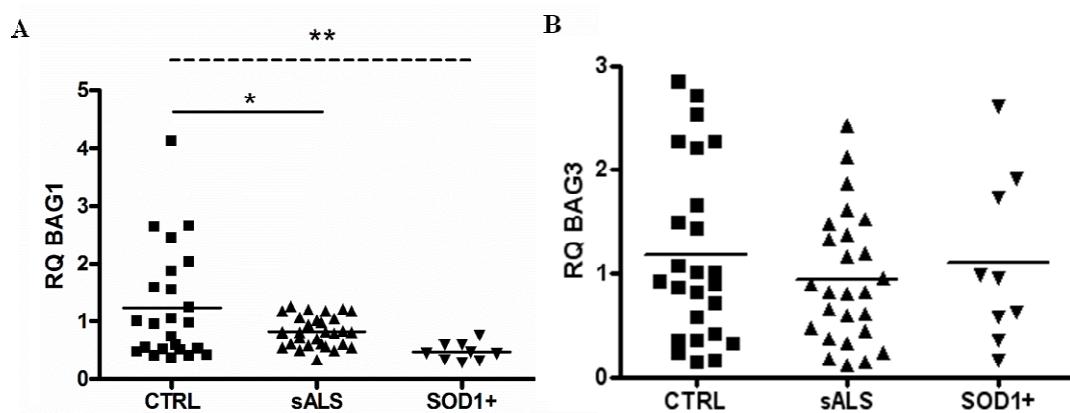


Figure 13

Gene expression levels of BAG1 (**A**) and BAG3 (**B**) in patients and controls. BAG1 mRNA levels were significantly reduced in both sporadic and SOD1+ ALS patients with respect to controls (* $p<0.05$; ** $p<0.01$). Relative quantification (RQ) of mRNA target is calculated as ratio to β -actin (CTRLs n=30; sALS n=30; SOD1+ n=9).



To better characterize the pathological mechanisms involving catabolic pathways could be very important to development pharmacological strategies to improve degradative systems efficiency and to facilitate removal of misfolded proteins. Based on eventual future results confirming an impairment of protein quality system, dedicated therapeutic strategies could be hypothesized to contrast the protein aggregation and accumulation which is a nodal event in ALS and in several other neurodegenerative diseases.

References

1999. A controlled trial of recombinant methionyl human BDNF in ALS: The BDNF Study Group (Phase III). *Neurology*. 52, 1427-33.
- Angelucci, F., et al., 2004. Transcranial magnetic stimulation and BDNF plasma levels in amyotrophic lateral sclerosis. *Neuroreport*. 15, 717-20.
- Arndt, V., et al., 2010. Chaperone-assisted selective autophagy is essential for muscle maintenance. *Curr Biol*. 20, 143-8.
- Barmada, S. J., et al., 2014. Autophagy induction enhances TDP43 turnover and survival in neuronal ALS models. *Nat Chem Biol*. 10, 677-85.
- Choi, J. S., et al., 2004. Co-chaperone CHIP associates with mutant Cu/Zn-superoxide dismutase proteins linked to familial amyotrophic lateral sclerosis and promotes their degradation by proteasomes. *Biochem Biophys Res Commun*. 321, 574-83.
- Crippa, V., et al., 2013. Differential autophagy power in the spinal cord and muscle of transgenic ALS mice. *Front Cell Neurosci*. 7, 234.
- Crippa, V., et al., 2010a. A role of small heat shock protein B8 (HspB8) in the autophagic removal of misfolded proteins responsible for neurodegenerative diseases. *Autophagy*. 6, 958-60.
- Crippa, V., et al., 2010b. The small heat shock protein B8 (HspB8) promotes autophagic removal of misfolded proteins involved in amyotrophic lateral sclerosis (ALS). *Hum Mol Genet*. 19, 3440-56.
- Cuervo, A. M., Dice, J. F., 2000a. Regulation of lamp2a levels in the lysosomal membrane. *Traffic*. 1, 570-83.
- Cuervo, A. M., Dice, J. F., 2000b. Unique properties of lamp2a compared to other lamp2 isoforms. *J Cell Sci*. 113 Pt 24, 4441-50.
- de Azevedo Cardoso, T., et al., 2014. Neurotrophic factors, clinical features and gender differences in depression. *Neurochem Res*. 39, 1571-8.
- Duberley, R. M., et al., 1997. Immunocytochemical studies of neurotrophins in cerebral motor cortex in Amyotrophic Lateral Sclerosis. *Brain Res*. 763, 259-63.
- Grundström, E., et al., 1999. Increased expression of glial cell line-derived neurotrophic factor mRNA in muscle biopsies from patients with amyotrophic lateral sclerosis. *J Neurol Sci*. 162, 169-73.
- Grundström, E., et al., 2000. GDNF but not BDNF is increased in cerebrospinal fluid in amyotrophic lateral sclerosis. *Neuroreport*. 11, 1781-3.
- Henderson, C. E., et al., 1993. Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature*. 363, 266-70.
- Huang, C. C., et al., 2014. Metabolism and mis-metabolism of the neuropathological signature protein TDP-43. *J Cell Sci*. 127, 3024-38.
- Ikeda, K., et al., 1995. Effects of brain-derived neurotrophic factor on motor dysfunction in wobbler mouse motor neuron disease. *Ann Neurol*. 37, 505-11.
- Ilzecka, J., Stelmasiak, Z., 2002. Brain-derived neurotrophic factor is not altered in the serum and cerebrospinal fluid of amyotrophic lateral sclerosis patients. *Neurol Sci*. 22, 473-4.
- Kaushik, S., et al., 2008. Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. *Mol Biol Cell*. 19, 2179-92.
- Kawamoto, Y., et al., 1998. Immunohistochemical localization of brain-derived neurotrophic factor in the spinal cords of amyotrophic lateral sclerosis and non-amyotrophic lateral sclerosis patients. *J Neuropathol Exp Neurol*. 57, 822-30.
- Kiffin, R., et al., 2004. Activation of chaperone-mediated autophagy during oxidative stress. *Mol Biol Cell*. 15, 4829-40.
- Kimpton, J., 2012. The brain derived neurotrophic factor and influences of stress in depression. *Psychiatr Danub*. 24 Suppl 1, S169-71.
- Koliatsos, V. E., et al., 1993. Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. *Neuron*. 10, 359-67.

- Küst, B. M., et al., 2002. Elevated levels of neurotrophins in human biceps brachii tissue of amyotrophic lateral sclerosis. *Exp Neurol.* 177, 419-27.
- Li, L., et al., 2008. Altered macroautophagy in the spinal cord of SOD1 mutant mice. *Autophagy.* 4, 290-3.
- Mitsumoto, H., et al., 1994. The effects of ciliary neurotrophic factor on motor dysfunction in wobbler mouse motor neuron disease. *Ann Neurol.* 36, 142-8.
- Molendijk, M. L., et al., 2014. Serum BDNF concentrations as peripheral manifestations of depression: evidence from a systematic review and meta-analyses on 179 associations (N=9484). *Mol Psychiatry.* 19, 791-800.
- Morimoto, N., et al., 2007. Increased autophagy in transgenic mice with a G93A mutant SOD1 gene. *Brain Res.* 1167, 112-7.
- Nishio, T., et al., 1998. Neutrophin switching in spinal motoneurons of amyotrophic lateral sclerosis. *Neuroreport.* 9, 1661-5.
- Novikova, L., et al., 1997. Effects of neurotransplants and BDNF on the survival and regeneration of injured adult spinal motoneurons. *Eur J Neurosci.* 9, 2774-7.
- Ochs, G., et al., 2000. A phase I/II trial of recombinant methionyl human brain derived neurotrophic factor administered by intrathecal infusion to patients with amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Other Motor Neuron Disord.* 1, 201-6.
- Pan, W., et al., 1998. Transport of brain-derived neurotrophic factor across the blood-brain barrier. *Neuropharmacology.* 37, 1553-61.
- Pluchino, N., et al., 2013. Steroid hormones and BDNF. *Neuroscience.* 239, 271-9.
- Sahu, R., et al., 2011. Microautophagy of cytosolic proteins by late endosomes. *Dev Cell.* 20, 131-9.
- Sartorius, A., et al., 2009. Correlations and discrepancies between serum and brain tissue levels of neurotrophins after electroconvulsive treatment in rats. *Pharmacopsychiatry.* 42, 270-6.
- Sasaki, S., 2011. Autophagy in spinal cord motor neurons in sporadic amyotrophic lateral sclerosis. *J Neuropathol Exp Neurol.* 70, 349-59.
- Sendtner, M., et al., 1992. Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature.* 360, 757-9.
- Song, C. Y., et al., 2012. Autophagy and Its Comprehensive Impact on ALS. *Int J Neurosci.* 122, 695-703.
- Song, Y., et al., 2013. Molecular chaperone Hsp110 rescues a vesicle transport defect produced by an ALS-associated mutant SOD1 protein in squid axoplasm. *Proc Natl Acad Sci U S A.* 110, 5428-33.
- Teixeira, A. L., et al., 2010. Circulating levels of brain-derived neurotrophic factor: correlation with mood, cognition and motor function. *Biomark Med.* 4, 871-87.
- Ulbricht, A., et al., 2013. Cellular mechanotransduction relies on tension-induced and chaperone-assisted autophagy. *Curr Biol.* 23, 430-5.
- Urushitani, M., et al., 2004. CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitininating Hsp/Hsc70. *J Neurochem.* 90, 231-44.
- Ventriglia, M., et al., 2013. Serum brain-derived neurotrophic factor levels in different neurological diseases. *Biomed Res Int.* 2013, 901082.
- Wang, J., et al., 2009. Progressive aggregation despite chaperone associations of a mutant SOD1-YFP in transgenic mice that develop ALS. *Proc Natl Acad Sci U S A.* 106, 1392-7.
- Watanabe, M., et al., 2001. Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues. *Neurobiol Dis.* 8, 933-41.
- Wong, A. S., et al., 2011. Molecular machinery of macroautophagy and its deregulation in diseases. *Biochim Biophys Acta.* 1812, 1490-7.
- Yang, Q., et al., 2009. Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy. *Science.* 323, 124-7.

Zetterström, P., et al., 2011. Proteins that bind to misfolded mutant superoxide dismutase-1 in spinal cords from transgenic amyotrophic lateral sclerosis (ALS) model mice. *J Biol Chem.* 286, 20130-6.

Zhang, C., et al., 2005. CHIP, a cochaperone/ubiquitin ligase that regulates protein quality control, is required for maximal cardioprotection after myocardial infarction in mice. *Am J Physiol Heart Circ Physiol.* 288, H2836-42.

Publications and Scientific Communications

Publications

BDNF SERUM LEVELS WITH RESPECT TO MULTIDIMENSIONAL ASSESSMENT IN ALS.

Tremolizzo L, Pellegrini A, Conti E, **Arosio A**, Gerardi F, Lunetta C, Magni P, Appollonio I, Ferrarese C
Neurodegenerative Diseases. 2015. *In press*

REDUCED EXPRESSION OF THE CHAPERONE-MEDIATED AUTOPHAGY CARRIER HSC70 PROTEIN IN LYMPHOMONOCYTES OF PATIENTS WITH PARKINSON'S DISEASE.

Sala G, Stefanoni G, **Arosio A**, Riva C, Melchionda L, Saracchi E, Fermi S, Brighina L, Ferrarese C.
Brain Res. 2013 Dec 19.

WHOLE-BLOOD GLOBAL DNA METHYLATION IS INCREASED IN AMYOTROPHIC LATERAL SCLEROSIS INDEPENDENTLY OF AGE OF ONSET.

Tremolizzo L, Messina P, Conti E, Sala G, Cecchi M, Airoldi L, Pastorelli R, Pupillo E, Bandettini Di Poggio M, Filosto M, Lunetta C, Agliardi C, Guerini F, Mandrioli J, Calvo A, Beghi E, Ferrarese C; EURALS Consortium.

Collaborators:

Cotelli MS, Corbo M, Maestri E, Georgouloupolou E, Marzorati L, Susani E, **Arosio A**, Chiò A, Fuda G, Pisa F.

Amyotroph Lateral Scler Frontotemporal Degener. 2013 Nov 13.

ROTENONE UPREGULATES ALPHA-SYNUCLEIN AND MYOCYTE ENHANCER FACTOR 2D INDEPENDENTLY FROM LYSOSOMAL DEGRADATION INHIBITION.

Sala G, **Arosio A**, Stefanoni G, Melchionda L, Riva C, Marinig D, Brighina L, Ferrarese C.
Biomed Res Int. 2013. Epub 2013 Jul 30.

Awards

THE BEST POSTER AWARD

MEF2D-MEF2C PATHWAYS ARE ALTERED IN LYMPHOMONOCYTES OF PATIENTS WITH SPORADIC AND FAMILIAL FORM OF AMYOTROPHIC LATERAL SCLEROSIS

Arosio A, Sala G, Gerardi F, Rodriguez-Menendez V, Lunetta C, Tremolizzo L, Ferrarese C
New perspectives in Neuroscience: Research results of Young Italian Neuroscientists, February 26, 2015;
Naples, Italy

Abstracts

MYOCYTE ENHACER FACTOR-2 PATHWAY IS ALTERED IN AMYOTROPHIC LATERAL SCLEROSIS PERIPHERAL BLOOD MONONUCLEAR CELLS

Tremolizzo L, **Arosio A**, Sala G, Rodriguez-Menendez V, Gerardi F, Lunetta C, Ferrarese C
XLVI Congress of the Italian Neurological Society, October 2015; Genoa, Italy

ALTERED miRNA IN AMYOTROPHIC LATERAL SCLEROSIS: SEARCHING FOR THE LOWEST COMMON MULTIPLE

Tremolizzo L, Loffreda A, **Arosio A**, Gerardi F, Lunetta C, Ferrarese C, Barabino S
XLVI Congress of the Italian Neurological Society, October 2015; Genoa, Italy

MYOCYTE ENHACER FACTOR 2 PATHWAY DISRUPTION IN LYMPHOMONOCYTES OF PATIENTS WITH SPORADIC AND FAMILIAL FORM OF AMYOTROPHIC LATERAL SCLEROSIS

Arosio A, Sala G, Gerardi F, Rodriguez-Menendez V, Lunetta C, Tremolizzo L, Ferrarese C
VI Meeting on the Molecular Mechanisms of Neurodegeneration Milan, Italy. May 28th-30th, 2015

ROtenone down-regulates HspA8/Hsc70 protein in vitro: a new possible toxic mechanism contributing to parkinson's disease

Sala G, Marinig D, Riva C, Colangelo A, **Arosio A**, Stefanoni G, Brighina L, Alberghina L, Ferrarese C
VI Meeting on the Molecular Mechanisms of Neurodegeneration Milan, Italy. May 28th-30th, 2015

MEF2D-MEF2C PATHWAYS ARE ALTERED IN LYMPHOMONOCYTES OF PATIENTS WITH SPORADIC AND FAMILIAL FORM OF AMYOTROPHIC LATERAL SCLEROSIS

Arosio A, Sala G, Gerardi F, Rodriguez-Menendez V, Lunetta C, Tremolizzo L, Ferrarese C

New perspectives in Neuroscience: Research results of Young Italian Neuroscientists, February 26, 2015; Naples, Italy

SERUM BDNF IN AMYOTROPHIC LATERAL SCLEROSIS DECREASES WITH DISEASE SEVERITY

Tremolizzo L, Pellegrini A, **Arosio A**, Conti E, Susani E, Lunetta C, Appollonio I; Ferrarese C

XLV CONGRESSO DELLA SOCIETÀ ITALIANA DI NEUROLOGIA, October 2014; Cagliari, Italy

MICRORNAs IN ALS: FROM MOLECULAR MECHANISMS TO CLINICAL RELEVANCE

Tremolizzo L, Loffreda A, **Arosio A**, Lunetta C, Ferrarese C, Barabino S

IX FENS Forum of Neuroscience 2014, 5-9 July 2014; Milan, Italy

EFFECT OF MITOCHONDRIAL COMPLEX I INHIBITION ON CHAPERONE-MEDIATED AUTOPHAGY AND MYOCYTE ENHANCER FACTOR 2D IN HUMAN NEUROBLASTOMA CELLS

Stefanoni G, Sala G, **Arosio A**, Riva C, Marinig D, Brighina L, Ferrarese C

Neurol Sci 2013; S34:S330

REDUCED EXPRESSION OF HSC70 PROTEIN IN LYMPHOMONOCYTES OF PATIENTS WITH PARKINSON'S DISEASE

Stefanoni G, Sala G, **Arosio A**, Riva C, Marinig D, Saracchi E, Brighina L, Ferrarese C

Neurol Sci 2013; S34:S329

STUDY OF ALPHA-SYNUCLEIN AND ITS CATABOLIC PATHWAYS IN LYMPHOMONOCYTES OF PATIENTS WITH PARKINSON'S DISEASE- Oral Symposium

Sala G, Stefanoni G, **Arosio A**, Riva C, Marinig D, Saracchi E, Fermi S, Brighina L, Ferrarese C

XV Congress of the Italian Society of Neuroscience (SINS), 3-5 October 2013; Rome, Italy

MYOCYTE ENHANCER FACTOR-2D EXPRESSION IN ALS LYMPHOMONOCYTES

Tremolizzo L, Sala G, Conti E, **Arosio A**, Rodriguez-Menendez V, Ferrarese C

XLIII CONGRESSO SOCIETÀ ITALIANA DI NEUROLOGIA, 6-9 October 2012;

DYSFUNCTION OF CHAPERONE-MEDIATED AUTOPHAGY IN LYMPHOMONOCYTES FROM PATIENTES WITH PARKINSON'S DISEASE AND IN A CELLULAR MODEL

Stefanoni G, Sala G, **Arosio A**, Melchionda L, Saracchi E, Fermi S, Brighina L, Ferrarese C

Neurol Sci 2011; S32:S546-547

CHAPERONE-MEDIATED AUTOPHAGY IN PARKINSON'S DISEASE: STUDY IN PERIPHERAL BLOOD CELLS FROM PATIENTS AND IN A CELLULAR MODEL

Stefanoni G, Sala G, **Arosio A**, Melchionda L, Saracchi E, Fermi S, Brighina L, Ferrarese C

Basal Ganglia 1 (2011) P43 159

ASSESSMENT OF CHAPERONE-MEDIATED AUTOPHAGY IN CIRCULATING LYMPHOMONOCYTES OF PATIENTS WITH PARKINSON'S DISEASE

Sala G, Stefanoni G, **Arosio A**, Melchionda L, Saracchi E, Fermi S, Brighina L, Ferrarese C

Fifth Meeting on the Molecular Mechanisms of Neurodegeneration Milan, Italy. May 13-15, 2011

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